Biomarkers of fat intake

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Biomarkers of fat intake

By

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A thesis submitted to King's College London for the degree of

Doctor of Philosophy in the Faculty of Science.

Diabetes and Nutritional Sciences Division

School of Medicine

King's College London

March 2013

For

Lady Fatimah Al-Zahra

Publications and presentations

Publications

Al-Hilal M, AlSaleh A, Maniou Z, Lewis F, Hall W, Sanders TAB and O'Dell SD (2013) Genetic variation at the *FADS1-FADS2* gene locus influences delta-5 desaturase activity and LC-PUFA proportions after fish oil supplement. *J Lipid Res* 54(2):542-551.

Abstract

Al-Hilal M. and Sanders T.A.B. (2012).Genetic variations in the *FADS1, FADS2* genes influence the proportions of long-chain n-6 polyunsaturated fatty acids in erythrocyte and plasma lipids. *Proc Nutr Soc* **71 (OCE2)**, E65.

Presentations

Fatty acid composition of adipose tissue as biomarker of fat intake in a Caucasian female twin (2010) at the 9th conference of the international society for the study of fatty acids and lipids (ISFFAL), Maastricht.

Abstract

The thesis reviews the previous use of biomarkers of fatty acid intake and concludes that there is a lack of supporting evidence from large randomized controlled trials (RCT) of sufficient duration for their use to be justified. The hypothesis that fatty acid biomarkers are robust indices of the intake certain fatty acids was tested by the analysis of blood samples from three large and long-term RCTs where dietary intake had been well controlled and compared with a control treatment. Erythrocyte lipid fatty acid composition was unable to detect changes in saturated fatty acid (SFA) or oleic acid intake. Plasma total lipids and phospholipids SFA were also poor indicators of SFA intake. The intake of *n*-3 long-chain polyunsaturated fatty acids (LC-PUFA) could be predicted from the proportions in plasma and erythrocytes. Principal components analysis appeared to be a valid data reduction technique to measure changes in fatty acid patterns. A co-twin study design conducted in 570 female participants enrolled in the St Thomas' Twins Study investigated the heritability of fatty acid biomarkers (adipose tissue and plasma). For most fatty acids, environmental factors (dietary intake) were dominant, but in the case of arachidonic acid, 65% of the variance was explained by additive genetic factors. Investigations subsequently explored the effects of variations in single nucleotide polymorphisms (SNP) in the fatty acid desaturase (*FADS*) genes on the fatty acid composition of the biomarkers. Polymorphism in *FADS1* rs174537 explained some of this variation. Carriage of the minor allele of rs174537 SNP also influenced the proportions of *n*-6 LC-PUFA in an RCT. Further research is suggested to identify what appeared to be a *FADS1*/*FADS2* haplotype predicting lower levels of LC-PUFA, which might be of public health significance.

In conclusion, plasma fatty acid composition can be recommended to elucidate the potential relationships between polyunsaturated, *trans*-unsaturated and branched chain fatty acid intake and non-communicable diseases.

Author's contribution

The author conducted the fatty acid analyses of adipose tissue from the TwinsUK study and plasma from TwinsUK, MARINA and CRESSIDA studies and all statistical analyses.

The author also planned and conducted a human intervention study aimed to investigate the acute and chronic effect of dietary palmitoleic acid using macadamia oil on markers of glucose and insulin sensitivity and cardiovascular risk factors. Unfortunately, the study failed to recruit the required number of subjects and thus was terminated. However, 6 subjects were recruited to the postprandial study and results from those are presented here. This study involved recruiting and screening subjects, handling and analysis of blood samples for plasma fatty acid proportions, glucose, insulin and plasma lipids (*see* **Appendix 1**).

Acknowledgements

I am deeply indebted to my supervisor, Prof. Tom Sanders, for his enthusiastic and invaluable supervision, academic support and guidance during the journey of this PhD. I would also like to extend my sincerest gratitude to Dr Sandra O'Dell for her great inspiration and support, providing a great amount of time and advice. I have been, indeed, very fortunate to work with her and Prof. Sanders.

I would also like to extend my sincerest thanks to Prof. Tim Spector and Prof. Chris Hammond for assistant and facilitating the TwinsUK study and the principal investigators of the MARINA and CRESSIDA studies for giving me the opportunity to work on these studies. I am grateful to Dr Fruhling Rijsdijk for training on using Mx for heritability analysis.

I would like to thank: the Kuwaiti Ministry of Health for funding my PhD; the special efforts of Dr Aseel Alsaleh for support with the statistical analysis of the MARINA study; Virginia Govoni for blood sampling; Robert Gray for technical support with laboratory analysis at KCL; Anne-Catherine Perz for help with I-LAB analysis and showing the muffin baking process; Rosie Calokatsia; Simon George, Jai David; Mary-Joe Searle, David Lincoln and Karen Conway for their continual assistance.

Special thanks to Dr Peter Milligan for his statistical advice; Dr Roy Sherwood, Tracy Dew in the Clinical Biochemistry Department at King's College Hospital for carrying out blood analyses; and Dianne Reidlinger for dietary analysis of the CRESSIDA study.

A select few people deserve thanks that are much harder to express; Catherine, Shouq, Androulla, Pei Ying, Hazreen, Sarah, Fawaz, Victoria, Filomena, Iyman, Somya, Stephanie, Anna, Monica and my best friend Bedoor Qabazard, for sharing special moments, and so many others have given me a tremendous amount of support, academic and otherwise, as well as some very fond memories.

I am entirely grateful to my family, Mum and Dad, brothers and sisters, nephews and nieces for all their encouragement and unconditional love! I am fortunate to have you around me and words cannot express my deepest love and gratitude to you.

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Abbreviations

AA	Arachidonic acid
ALA	α-linolenic acid
ANCOVA	Analysis of covariance
apoB	ApolipoproteinB
AT	Adipose tissue
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesteryl ester transferase
CHD	Coronary heart disease
CHO	Carbohydrate
CI	Confidence interval
COX1	Cyclo-oxygenases type 1
COX2	Cyclo-oxygenases type 2
CV	Coefficients of variance
CVD	Cardiovascular disease
D5D	Delta-5 desaturase
DHA	Docosahexaenoic acid
DGLA	Dihomo-gammalinolenic acid
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid
DRV	Dietary reference value
DZ	Dizygotic
EFA	Essential fatty acids
EPA	Eicosapentaenoic acid
FFQ	Food frequency questionnaire
FH	Familial hypercholesterolaemai
GC	Gas chromatography
GM	Geometric mean
HDL-C	High density lipoprotein
HM	High-MUFA
HOMA-IR	Homeostatic model assessment of insulin resistance
HS	High saturated fat

IDF	International Diabetes Federation
IDL	Intermediate density lipoprotein
IL-6	Interleukin-6
IOM	Institute of medicine
GLA	Gama linolenic acid
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acids
LD	Linkage disequilibrium
LDL	Low density lipoprotein
MAF	Minor allele frequency
MARINA	Modulation of Atherosclerosis Risk by Increasing dose of N-3 fatty Acids
MUFA	Monounsaturated fatty acids
MZ	Monozygotic
NCBI	National Centre for Biotechnology Information
NCEP ATP III	National Cholesterol Education Program's Adult Treatment Panel
NEFA	Non-esterified fatty acids
NDNS	National diet and nutrition survey
P:S	Polyunsaturated: saturated fat ratio
PCA	Principal component analysis
PL	Phospholipid
PPAR	Peroxisome proliferator-activated receptor alpha
PUFA	Polyunsaturated fatty acids
RISCK	Reading, Imperial, Surrey, Cambridge, King's
SD	Standard deviation
SFA	Saturated fatty acid
SNP	Single nucleotide polymorphisms
SPH	Sphingomyelin
T2DM	Type 2 diabetes mellitus
TAG	Triacylglycerol
TC	Total cholesterol
TFA	*Trans* fatty acid
TNF-α	Tumour necrosis factor-α
VLDL	Very low density lipoprotein
WHO	World Health Organization
M/F	Male/female

Chapter 1: Introduction

Dietary fat intake may be associated with cardiovascular disease but the relationship is marred by the limitations in the methods used to assess both the total fat intake and the type of fat consumed. Assessment of dietary intake of fat using traditional methods such as food frequency questionnaire is prone to a number of limitations, which may lead to unreliable assumptions about diet-disease relationships. Biomarkers could provide a potential robust alternative to assess dietary intake of certain types of fatty acids. Depending on the turnover of the lipid pool, biomarkers could be used to detect short, medium or long-term intake of fat. However, it is uncertain to what extent they are influenced by genetic factors. The work described tests some of the assumptions from prospective cohort studies by comparing changes in biomarkers in large well-controlled intervention studies with those predicted from accurate measurement of dietary intake. Further comparisons of biomarkers are made according to single nucleotide polymorphisms in the desaturase enzymes (*FADS1*, *FADS2* and *SCD1*). The effect of additive genetics influencing adipose tissue and plasma biomarkers of fat intake were estimated using linear structural equation modelling based on analysis of biomarkers in pairs of twins. In order to preface this work, a brief introduction on nutritional biochemistry of fat, a summary of present knowledge with regard to different fatty acids and risk of cardiovascular disease, and a review of previous research on biomarkers of fat intake are presented.

1.1 Chemistry and types of fat

Lipids are fatty acids, esters and potential esters of fatty acids (Christie, 2010). Lipids can be separated into fatty acids, glycerolipids (mono-, di- and tri-acylglycerols), glycerophospholipids (e.g. phosphatidylcholine (lecithin), phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol), ceramides, sphingolipids, sterol lipids (e.g. cholesterol, cholesteryl esters), prenol lipids, glycolipids, waxes and polyketides. Lipids are often divided into simple and complex lipids: simple lipids include fatty acids, neutral fats (e.g. triacylglycerols) and waxes (e.g. esters of fatty acids); complex lipids include ceramides, phosphoglycerides, plasmalogens, sphingolipids, glycolipids, steroids and sterol ester (Christie, 2010; Fahy *et al*. 2005).

From a nutritional standpoint, fatty acids and glycerolipids are concentrated sources of energy and the main energy store in animals. An Atwater factor of 9 kcal/g (37 kJ) is usually applied but the energy yield does vary with chain length but short (C2-C6) and medium chain (C6-12) length fatty acids yield 7 kcal/g. However, most fats contain C16-C18 fatty acids and food tables assume each gram of fat yields 9 kcal/g (37 kJ), compared to 4 and 3.75 kcal/g for protein and carbohydrate (CHO) respectively (WHO/FAO, 2010). Dietary fat is a mixture of triacylglycerols (TAG), where the three fatty acids are attached to a glycerol backbone in positions *sn*-1, *sn*-2 or *sn*-3 (**Figure 1.1**). Most fatty acids are even number, straight chain molecules having a carboxyl group (COOH) at the proximal end and a terminal methyl group (CH3) at the other. Fatty acids are classified according to their chain length, degree and geometry of un-

saturation. A standardized shorthand nomenclature is used where the number of carbons in the molecule is given followed by a colon and the number of double bonds in the fatty acids with information about the positions of the double bonds from the carboxyl end (**Table 1.1**). For series of fatty acids derived from one of the parent unsaturated fatty acids, the suffix of *n*-x is more commonly used in nutritional sciences: where x is the number of carbons from the terminal (omega) methyl group. The older term omega-3 and omega-6 is still widely used to refer to members of the *n*-3 and *n*-6 series of polyunsaturated fatty acids (PUFA). Depending on the length of carbon chain, fatty acids are divided into short chain (<7 carbons), medium chain (8-13 carbons), long chain (14-18 carbons) and very long chain fatty acids (>20 carbons) (Ratnayake & Galli, 2009); lauric acid (12:0) is sometimes included with long-chain fatty acids. Saturated fatty acids (SFA) contain single bond linkage between each carbon atom and most are straight rather branched chain as shown in **Figure 1.2**. At room temperature (22˚C), short-chain fatty acids are volatile; medium-chain fatty acids are liquid, whereas long-chain SFA are solid. The desaturase enzymes insert double bonds in the *cis*-configuration where the hydrogen molecules are on the same side of the double bond and this creates a kink in the molecule of the fatty acids, which lowers the melting point considerably. Unsaturated fatty acids have one (monounsaturated; MUFA) or more (polyunsaturated; PUFA) double bonds in their structure. Dietary fats are mixtures of fatty acids but are described as saturated, monounsaturated or polyunsaturated based on the dominant proportion of fatty acid present. Animal fats contains predominantly a mixture of MUFA, mainly oleic acid (18:1*n*-9), and SFA predominantly palmitic (16:0)

and smaller amounts of myristic (14:0) and stearic (18:0) acids, the proportion of stearic acid being higher in the fat from ruminant animals. The amount of PUFA present in animal fat depends on the amount in the diet in monogastric food animals (poultry, swine, rabbits), but is always very low (<3%) in fat from ruminants (goats, sheep, cattle) because of biohydrogenation of PUFA in the rumen (Christie, 2010).

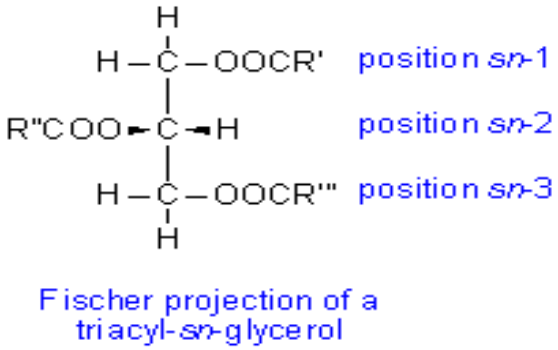


Figure 1.1 Structure of a triacylglycerol (The lipid library - http://lipidlibrary.aocs.org/) [accessed March 2013]

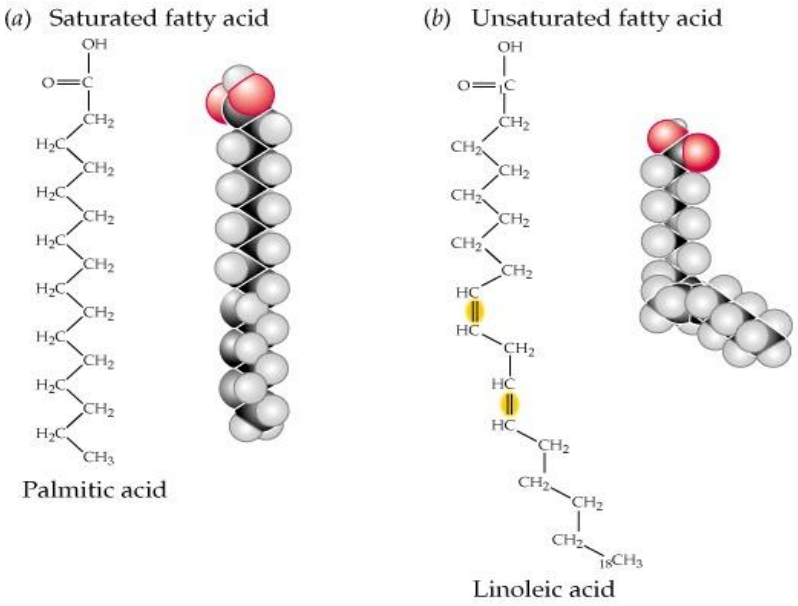


Figure 1.2 Structure of saturated and unsaturated fatty acid. From

(http://newberrybiology.wordpress.com/2011/02/09/hydrophobia) [accessed on March, 2013]

Table 1.1 Main fatty acids in the diet.

Systematic nomenclature	Common name	Short hand notation
Saturated fatty acids (SFA)		
Ethanoic acid	Acetic acid	2:0
Butanoic acid	Butyric acid	4:0
Hexanoic acid	Caproic acid	6:0
Octanoic acid	Caprylic acid	8:0
Decanoic acid	Capric acid	10:0
Dodecanoic acid	Lauric acid	12:0
Tetradecanoic acid	Myristic acid	14:0
Pentadecanoic acid	Pentadeciyic acid	15:0
Hexadecanoic acid	Palmitic acid	16:0
Heptadecanoic acid	Margaric acid	17:0
Octadecanoic acid	Stearic acid	18:0
Eicosanoic acid	Arachidic acid	20:0
Cis-monounsaturated fatty acids (*cis*-MUFA)		
Hexadecenoic acid	Palmitoleic acid	16:1Δ9c (18:1*n*-7)
Octadecenoic acid	Oleic acid	18:1Δ9c (18:1*n*-9)
Octadecenoic acid	Petroselinic acid	18:1Δ11t (18:1n-7)
Eicosenoic acid	Gadoleic acid	20:1Δ9c (20:1*n*-11
Docosaenoic acid	Erucic acid	22:1Δ13c (22:1*n*-9)
Docosaenoic acid	Cetoleic acid	22:1 Δ11c (22:1*n*-11)
Trans-monounsaturated fatty acids (*trans*-MUFA)		
Octadecenoic acid	Elaidic acid	18:1Δ9t (18:1*n*-9 trans)
Octadecenoic acid	*Trans-vaccenic acid*	18:1Δ11t (18:1*n*-7 trans)
Octadecenoic acid	*Vaccenic acid*	18:11Δ11t (18:1*n*-7)
Polyunsaturated fatty acids (PUFA)		
n-6 Polyunsaturated fatty acids (omega-6 PUFA)		
Octadecadienoic acid	Linoleic acid	18:2Δ9c,12c (18:2*n*-6)
Octadecatrienoic acid	γ-Linolenic acid	18:3Δ6c,9c,12c (18:3*n*-6)
Eicosatrienoic acid	Dihomo- γ-linolenic acid	20:3 8c,11c,14c (20:3*n*-6)
Eicosatetraenoic acid	Arachidonic acid	20:4Δ5c,8c,11c,14c (20:4*n*-6)
Docosatetraenoic acid	Adrenic acid	22: 4Δ7c,10c,13c,16c (22:4*n*-6)
Docosapentaenoic acid	Osbond acid	22: 4Δ 4c, 7c,10c,13c,16c (22:5*n*-6)
n-3 Polyunsaturated fatty acids (omega-3 PUFA)		
Octadecatrienoic acid	α-Linolenic acid	18:3Δ9c,12c,15c (18:3*n*-3)
Octadecatetraenoic acid	Stearidonic acid	18:4Δ6c, 9c,12c,15c (18:4*n*-3)
Eicosapentaenoic acid	Timnodonic acid	20:5Δ5c,8c,11c,14c,17c (20:5*n*-3)
Docosapentaenoic acid	Clupanodonic acid	22:5Δ7c,10c,13c,16c,19c (22:5*n*-3)
Docosahexaenoic acid	Cervonic acid	22:6Δ4c,7c,10c,13c,16c,19c(22:6*n*-3)
Conjugated linoleic acid (CLA)		
Octadecadienoic acid	Rumenic acid	18:2Δ9c,11t
Octadecadienoic acid		18:2Δ10t,12c

The coconut (*Cocos nucifera*) and the kernel of the oil palm (*Elaeis guineensis* and *Elaeis oleifera*) contain high proportions of the medium chain fatty acids caprylic, capric and lauric acids, but very low levels of PUFA (<2%). Palm oil, which comes from the mesocarp of the palm fruit, contains about 42% palmitic acid, 37% oleic acid, 10% linoleic acid and 5% stearic acid. It has a symmetrical triacylglycerol structures with the SFA at positions *sn*-1 and *sn*-3. Cocoa butter, which is the fat from the cocoa bean (*Theobroma cacao*), has a similar symmetrical structure but higher levels of stearic acid and less palmitic acid but is low in PUFA (<3%). Related tropical oils that share this symmetrical TAG structure are highly valued as cocoa butter equivalents for chocolate confectionary and include shea butter (*Butyrospermum parkii*), sal (*Shorea robusta*), kokum gurgi (*Garcinia indica*), illipe (*Shorea spp*) and mango kernel (*Mangifera indica*) oils. Most of the major seed oils; soyabean (*Glycine max)*, rapeseed (*Brassica napus*), sunflower (*Helianthus annuus*), and corn (*Zea mays*) contain low levels of stearic acid unless hydrogenated. Plants seeds oils are generally high in MUFA and/or PUFA. Rapeseed and olive oil (*Olea europaea*) have a high content of MUFA but newer varieties of sunflower (high oleic sunflower oil) and soybean (mid-oleic soybean oil) oils contain less linoleic acid and more oleic acid are also high in MUFA. Linoleic acid (18:2*n*-6; LA) is the dominant PUFA in seed oils and contains less than 1% alpha-linolenic acid (18:3*n*-3; ALA) with the exception of soybean and rapeseed oils. A few exotic oils such as evening primrose (*Oenothera biennis*), borage (*Borago officinalis*) and hemp seed (*Cannabis sativa*) oils contain gamma-linolenic (18:3*n-6*; GLA) and stearadonic acid (18:4*n-3*) (Christie, 2010).

Ruminant animals (sheep, cows, goats) have a gut microbiota that biohydrogenate linoleic and **α**-linolenic acid present in diet mainly to stearic acid and C18 *trans* isomeric fatty acids (TFA) – levels are in the range of 3-7% of the total fatty acids (Sebedio & Christie, 1998). Higher levels of TFA are found in industrially processed fats particularly arising from the partial hydrogenation with a nickel or copper catalyst, where the proportion of TFA can be as high as 50%. Fully hydrogenated fats, however, contain no TFA. Smaller amounts of TFA can be formed during the steam deodorisation of fats particularly if temperatures are not correctly controlled. Levels of TFA as high as 4-5% have been observed in rapeseed oil exposed to high temperature deodorisation (Ackman & Mag, 1998). However, under good manufacturing practice levels of TFA should be below 1%. TFA, though, are not formed in a significant amounts during cooking processes such as deep-fat frying (WHO/FAO, 2010).

LA and ALA are considered essential nutrients because they cannot be synthesized *de novo* and are required for normal physiological functioning. Animals cannot synthesize them is because they lack the desaturase enzymes, which are present in plants, that insert double bond beyond 6-carbons from the terminal methyl group. Both LA and ALA, however, can undergo further desaturation towards the carboxyl end and chain elongation to form two distinct series of metabolites. Both LA and ALA give rise to parallel series of metabolites, *n*-6 and *n*-3 respectively (*see* **Figure 1.3**). Arachidonic acid (20:4*n*-6; AA) and docosahexaenoic acid (22:6*n*-3; DHA) both have unique physiological roles (WHO/FAO, 2010). The long-chain PUFA (LC-PUFA) of both *n*-6

and *n*-3 series play an important role in membrane function, but it is the metabolites of AA that have received most attention because of their key role in mediating thrombosis and inflammation. Metabolites of AA and dihomo-gamma-linolenic acid (20:3*n*-6; DGLA) are involved, as second messengers, in cell signalling. Phospholipids containing AA in particular are important in this respect. AA when released from phospholipids can undergo oxidation and molecular rearrangement to form signalling molecules such as prostaglandins but partial glycerides containing arachidonic acid and anandamide are also physiologically important ligands for the endocannabinoid receptor (Pertwee *et al.* 2010). When released from phospholipids, AA is oxidized by cyclo-oxygenase to form an endoperoxide which is subsequently rearranged according to tissue to give rise to various daughter compounds such as classical 2- series prostaglandins e.g. PGE_2, $PGF_{1-\alpha}$, and thromboxane TXA_2 in platelets and prostacyclin PGI_2 in endothelial cells. It can also act as a substrate for 5-lipooxygenase which gives rise to the 4-series leukotrienes (notably LTB_4) which is a chemotactic molecule involve in bronchoconstriction. DGLA gives rise to the 1-series prostanoids but does not give rise to a thromboxane, prostacyclin or leukotrienes. Eicosapentaenoic acid (20:5*n*-3; EPA) can give rise to a parallel series of cyclo-oxygenase and lipoxygenase metabolites: the 3-series prostanoids (PGE_3, TXA_3, PGI_3) and 5-series leukotrienes (LTB_5). However, these metabolites with the exception of PGI_3 are generally inactive or less active than those derived from AA. Under normal circumstances, the main endpoint of ALA metabolism is DHA. DHA plays an important role in visual function (WHO/FAO; 2010). DHA is not a substrate for cyclo-oxygenase but can act as a competitive inhibitor (Sanders,

1988). More recently, Serhan *et al*. (2004) have described other metabolites of 20-22 carbon PUFA called resolvins and neuroprotectins and some of these molecules can be derived from DHA but their exact physiological role remains at present uncertain. Collectively these metabolites are called eicosanoids and docosanoids and are signalling molecules that have important local biological activities at low concentrations (Calder, 2006).

Cyclo-oxygenase metabolites of AA are essential for normal physiological functioning in a variety of tissues and a failure to produce these metabolites explains reproductive failure, impaired platelet aggregation and impaired immune function in essential fatty acid deficiency (Sanders, 1988). Overproduction of cyclo-oxygenase metabolites can occur resulting in inflammation and this is usually a consequence of increased expression of the inducible cyclo-oxygenase-2 (COX-2) enzyme rather than the constitutive cyclo-oxygenase-1 (COX-1) enzyme. Cyclo-oxygenase is the target for most non-steroidal anti-inflammatory drugs (NSAID) such as aspirin, ibuprofen and paracetamol. Low dose aspirin is a powerfull inhibitor of platelet thromboxane A_2 production from AA and is widely used at a dose of 75mg/d to prevent second heart attack and stroke. However, a major side effect of aspirin is gastric irritation, which would indicate a protective effect of prostaglandins on the stomach, and an increased risk of intestinal haemorrhage. COX-2 inhibitors were developed, which do not cause gastritis, as anti-inflammatory agents for pain relief in rheumatism and other inflammatory disorders. However, their use has been associated with an increased rather

than a reduced risk of cardiovascular disease (CVD) (Drazen, 2005). The Nobel Laureate Sir John Vane, who was awarded the prize for his work on aspirin, suggested that replacing AA by EPA in membrane phospholipids would alter the balance of active eicosanoids and, therefore, have anti-thombotic and anti-inflammatory effects (Dyerberg *et al*. 1978). He proposed that this might explain the relatively low incidence of inflammatory disorders in some populations that consume very large amounts of fish or marine mammals as documented by Bang *et al*. (1980). However, later research showed that the levels of intake of EPA and DHA required to produce these anti-thombotic and anti-inflammatory effects are usually well in excess of 3g/d and in the case of Greenland Inuit it was in the range of 5-14g/d (Bang *et al.* 1980). In most populations, the levels of intake of EPA and DHA are usually well below 1g/d (WHO/FAO, 2010). There are many studies in animals that clearly demonstrate decreased risk of thrombosis and inflammation when fed diets high in fish oil, rich in a mixture of EPA and DHA and low in LA (Sanders, 1988; Calder 2006). It is also possible to produce similar effects in animals by restricting LA intake and increasing the intake of ALA (Sanders, 1988). However, human studies have found that dietary supplementation with ALA only leads to a small increase in EPA and no changes in DHA in blood (Sanderson *et al*. 2002). Stable isotope studies also suggest that the rate of synthesis of DHA from ALA is low (Burdge *et al.* 2004; Goyens *et al*. 2006). In contrast, even small amounts of dietary DHA result in pronounced increases in blood levels of DHA. This has led some to suggest that the human ability to make ALA from DHA is limited and that preformed DHA may be desirable in the diet (Calder, 2006).

However, for most populations dietary fat intake is derived from plant oils and the fat from land animals and vegetarians who consume little preformed DHA appear to be in good health (Key *et al*. 2009)

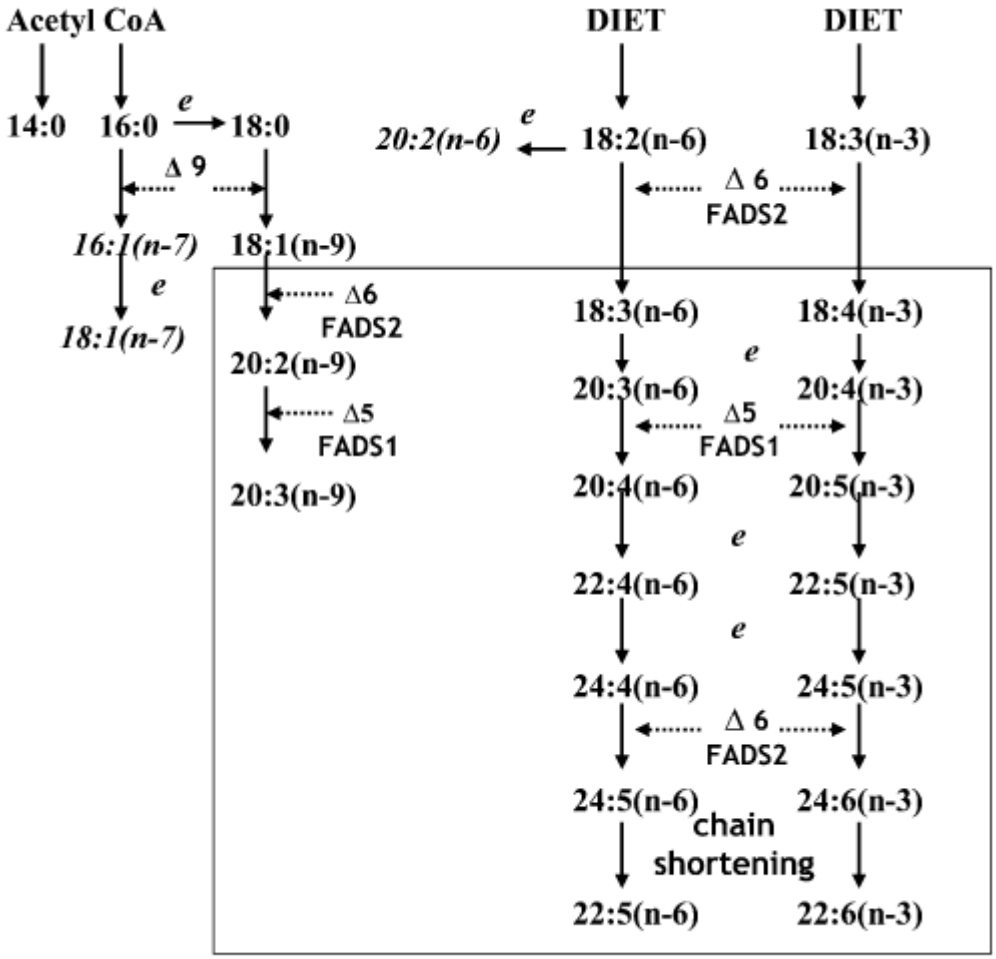


Figure 1.3 Metabolic pathway of n-3, n-6, and n-9 fatty acids in mammals. From (Xie & Innis, 2008)

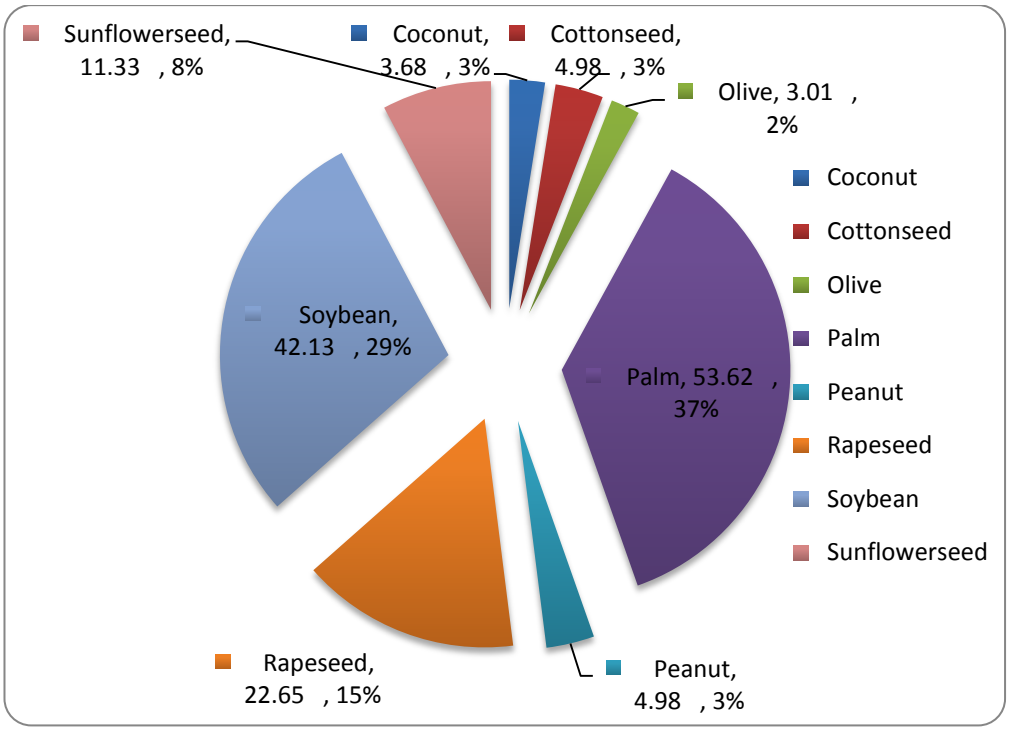


Figure 1.4 Global vegetable oil production in millions of metric tons. Source: USDA Economic, Statistics and Market Information (http://usda.mannlib.cornell.edu) [Accessed 04/03/2013].

1.2 Global food sources of oils and oil crops

Palm oil is currently the major vegetable oil produced globally followed by soybean oil and then rapeseed and sunflower seed oils (**Figure1.4**). Most vegetable oil is used for food but some is used in cosmetics and in biodiesel. Palm oil is produced mainly in Indonesia and Malaysia and to a lesser extent in West Africa (WHO/FAO, 2010). While crude red palm oil which is rich in carotene is consumed in West Africa, most of the

Indonesian and Malaysian palm oil is physically refined removing the carotenoids and fractionated into palm stearin, palm olein, palm mid-fraction and super palm olein which have decreasing levels of SFA. Palm stearin (65% SFA) is a high value product used in blend to provide the solid fat component of margarine and palm olein mid-fraction (mainly 1-3 dipalmitoyl, 2-oleyl glycerol). It is also a high value product used as a cocoa butter substitute (Marangoni *et al*. 1995). The cheaper and more abundant palm olein contains about 45% SFA, whereas super-palm olein (mainly 1 palmitoyl, 2-3 dioleyl glycerols) contains about 35% SFA. Both palm olein and super palm olein are liquid at room temperature. Palm olein has a melting point around 14°C in contrast to coconut oil, which tends to be solid at room temperature. Soybean oil is mainly produced in the United States and South America. Most of the olive oil production is in the Mediterranean basin, but olive cultivation is increasingly established in other countries with a Mediterranean climate. Most other seed oils, such as sunflower and corn oils, also require strong sunlight to be grown and do not flourish in temperate climates. In contrast, rapeseed and linseed grow well in temperate climates such as Canada and Northern Europe (WHO/FAO, 2010)

A substantial amount of vegetable oil is used by the food industry to make processed foods such as margarine, cakes, biscuits and snack foods such as crisps. The type of fat used is usually dependent upon the market price of the fat being used (blend flexibility describe the ability to change the oils used depending on price) and the physical characteristics required for the food. Some high melting point fats are required in bakery

products and chocolate confectionary as oils with a low melting point make inferior products. Many food manufacturers also specify the use of vegetable oil rather than cheaper animal fats in their products so that they are acceptable to consumers in markets where derivatives of beef and pork are not acceptable for religious reasons. There is also increasing demand for products that are acceptable to vegetarians. This generally has led to increased use of fats with higher LA content and there is evidence to show that the intake of LA has increased in many Western European countries, North America. In contrast, the use of animal fats used for these purposes has fallen as shall be discussed in later sections. Historically, intakes of LA have been high in the Middle East owing to the traditional use of oils such as sunflower and safflower oil (Cobley, 1976).

Animal fats make a substantial contribution to fat intake in economically developed countries. This fat intake is mainly derived from carcass meat and from milk and its products. The composition of these fats is far less variable and so risk of errors in estimation of intake from these sources is low. However, the levels of fat in meat can vary with production methods and it is necessary to take account of fat discarded during butchering, food preparation and plate waste. Processed meat products generally have a higher fat content than carcass meat and foods such as sausages may contain about a third of their weight as fat (McCance & Widdowson, 1998). Ruminants (cattle, goats, sheep, and camels) produce most milk for human consumption and butter fat with a high SFA content (63%) with only small amounts of PUFA (**Table 1.2**). Meat fat typically contains about 45% SFA and that from ruminant animals contains more stearic

acid and only small amounts of PUFA. The fat from monogastric animals (pigs and chickens) contains higher levels of PUFA (about 10% of the fatty acids) because the dietary PUFA are not biohydrogenated by rumen bacteria and lamb and beef typically contains less than 3% PUFA. Fish only provide small amounts of fat in most diets but the oil from fish contains relatively high amounts of *n*-3 LC-PUFAs (EPA and DHA). These *n*-3 LC-PUFA are synthesised by marine algae (Metz *et al.* 2001) and accumulate up the food chain with the highest amounts being found in predator fish e.g. tuna, mackerel and salmon (WHO/FAO, 2010).

Table 1.2 Fatty acid composition (wt %) of some edible oils and fats (ESFA, 2010)

	Butter fat	Coconut oil	Palm kernel oil	Palm oil	Cocoa butter	Olive oil	Rapeseed oil	Sunflower oil	Corn oil	Soybean oil
SFA	63.4	86.5	81.5	49.3	59.7	13.8	7.4	10.3	12.9	15.3
<12:0	11	14.1	7.2	0	0	0	0	0	0	0
12:0	3.2	44.6	47	0.1	0	0	0	0	0	0
14:0	9.1	16.8	16.4	1	0.1	0	0	0	0	0
16:0	26.8	8.2	8.1	43.5	25.4	11.3	4.3	5.9	10.6	10.7
18:0	12.3	2.8	2.8	4.3	33.2	2	2.1	4.5	1.8	4
MUFA	25.9	5.8	11.4	37	32.9	73	63.3	19.5	27.6	22.7
18:1*n*-9	21	5.8	11.4	36.6	32.6	71.3	61.7	19.5	27.3	22.6
Trans-18:1	3.7									
PUFA	3.7	1.8	1.6	9.3	3	10.5	28.1	65.7	54.7	57.3
18:2 *n*-6	3.3	1.8	1.6	9.1	2.8	9.8	18.6	65.7	53.2	50.1
18:3 *n*-3	0.4	0	0	0.2	0.1	0.8	9.1	NA	1.2	6.5

1.3 Dietary sources of essential fatty acids (EFAs)

Both LA and ALA are essential nutrients and a lack of these PUFA causes essential fatty acid (EFA) deficiency, a condition first described in rats (Burr & Burr, 1929) but later described in human infants and adults undergoing parenteral nutrition. Classical EFA deficiency, which occurs when the intake of LA falls below 1% energy, is unlikely to occur among individuals self-selecting their diets because even the most meagre diets provide more than this. The manifestations of EFA deficiency include a scaly skin with dryness, increased water loss throughout the skin, poor growth and impaired energy utilisation. Deficiency is regarded as iatrogenic and was first reported in infants fed skimmed milk and later adults receiving fat-free intravenous nutrition. It was estimated that about 1% of the energy as LA is sufficient to prevent classical EFA deficiency. The essentiality of ALA hinges on its conversion to DHA, which is an important component of the retinal membranes and the brain. Where DHA is replaced by osbond acid (22:5*n*-6) impairment of visual function and learning have been reported. There is some debate concerning the human capacity to convert ALA to DHA but the requirements for visual function can be met by ALA alone (WHO/FAO, 2010). Estimates for the minimum requirements of ALA suggest a minimum intake of 0.2% energy (Department of Health, 1991). Human milk contains varying amounts of PUFA depending on the maternal diet but on average contains about 8%-10% PUFA. In the past milk formula were based around cow's milk and had a low PUFA content (<2%), but then it became fashionable to replace cow's milk fat with vegetable oil because butter fat was not as well digested.

This resulted in higher levels of LA. Modern infant breastmilk substitutes are formulate to mimic breastmilk using: enzymically modified palm oil (Betapol™), where the palmitic acid is in the *sn*-2 position, to improve fat digestibility; vegetable oils to supply LA and ALA (typically in a ratio of 5:1); single cell oils/egg lecithin to provide AA and DHA (WHO/FAO 2010).

Exposure to high temperatures, metals such as copper and iron and oxygen results in oxidation of PUFA and this increases with the number of double bonds in the molecule. For this reason, food processors often select oils with low levels of ALA where heat treatment might lead to oxidative rancidity especially for deep frying applications. Oils used are partially hydrogenated to reduce their ALA content and thus improve their stability to oxidation. It has been argued that the consumption of *n*-3 fatty acids was considerably high in early human diets. However, industrialization and fast foods development diminish the content of *n*-3 fatty acids and amplify those of *n*-6 in the human diet. It has been argued that early human beings, who were hunter gatherers, would have had a low *n*-6:*n*-3 ratio in their diet. Nowadays, the ratio is much higher because of the inclusion of high sources of *n*-6 rich vegetable oils in the diet and this may have adverse health consequences (Simopoulos, 2011). Some researchers blame this change for the increase in many diseases such as CVD, cancer and inflammatory diseases (Das, 2008). However, there are other more plausible factors to explain these changes such as tobacco and alcohol use, physical inactivity and obesity. Furthermore, over the past fifty years where intakes of LA have increased there has been a continuing

pattern for increased life-expectancy and in the UK and many Western European countries. For example, deaths from CHD under the age 75 have fallen by over 50% in the last 10 years (Smolina *et al*. 2012). The dietary reference intakes for PUFA are substantially high than the amounts to prevent EFA deficiency and these recommended intakes are based on the association of the quality of fat intake with risk of CVD which shall be discussed in the following section.

1.4 Cardiovascular disease

CVD is a term that refers to arterial diseases affecting the heart and circulatory system: the major forms of CVD are coronary heart disease (CHD) or ischemic heart disease (IHD), which affects the heart, cerebrovascular disease/stroke, which affects the brain and peripheral vascular disease, which affects the limbs and results in claudication. A common feature in the pathology of these disorders is atherosclerosis, which is a nodular thickening and hardening of the arterial wall resulting in cholesterol rich lesions called atheromatous plaques. These lesions narrow the lumen of medium and large arteries and if they rupture trigger thrombosis which results in a clinical event i.e. heart attack, stroke or claudication (Warrell *et al*. 2010).

1.4.1 Incidence and prevalence of CVD

CVD is the leading-cause of death worldwide. However, there is great variation in the rate of death from the different forms of CVD between countries according to the WHO

MONICA study (Tunstall-Pedoe *et al*. 2000). Stroke rates tend to be more strongly associated with elevated blood pressure than atherosclerosis, whereas CHD is more strongly associated with atherosclerosis. Generally, rates of stroke are higher than that of CHD in the Far East. The lowest rates of CHD are in Japan and some of the Southern Mediterranean countries and the highest are now in Eastern European countries. Mortality from CVD has been falling steadily in Western Europe over the past 30 years. In the UK, CVD accounts for 36% of premature deaths (aged <75 years) in men and 27% in women according to the British Heart Foundation (www.bhf.org.uk). Recent data suggest that CVD death rates have fallen by over 50% in the last 10 years (Smolina *et al*. 2012). The reasons for the fall are uncertain but are thought to be due to better treatment, smoking cessation and a secular fall in blood pressure and serum cholesterol despite an increase in the prevalence of diabetes and obesity (Bajekal *et al*.2012). Dietary fat has been linked to risk of CVD mainly, but not exclusively, through its effects on blood lipids.

1.4.2 Risk Factors for cardiovascular diseases

CVD risk factors can be split into modifiable and un-modifiable risk factors (age, gender and genes). Modifiable factors include elevated serum cholesterol concentrations and a low high density lipoprotein cholesterol (HDL-C) which are best indicated by a high total cholesterol:HDL-C ratio (TC:HDL-C) (Lewington *et al.* 2007), cigarette smoking, high blood pressure (BP), type-2 diabetes mellitus and physical inactivity. Measures of body mass index (BMI) or waist add little in the way of risk prediction

when their effects on blood lipids, risk of diabetes and BP are taken into account. However, obesity is important in causing these abnormalities and there is robust evidence showing an almost linear increase in risk of stroke and IHD mortality as BMI increases (**Figure 1.5)**.

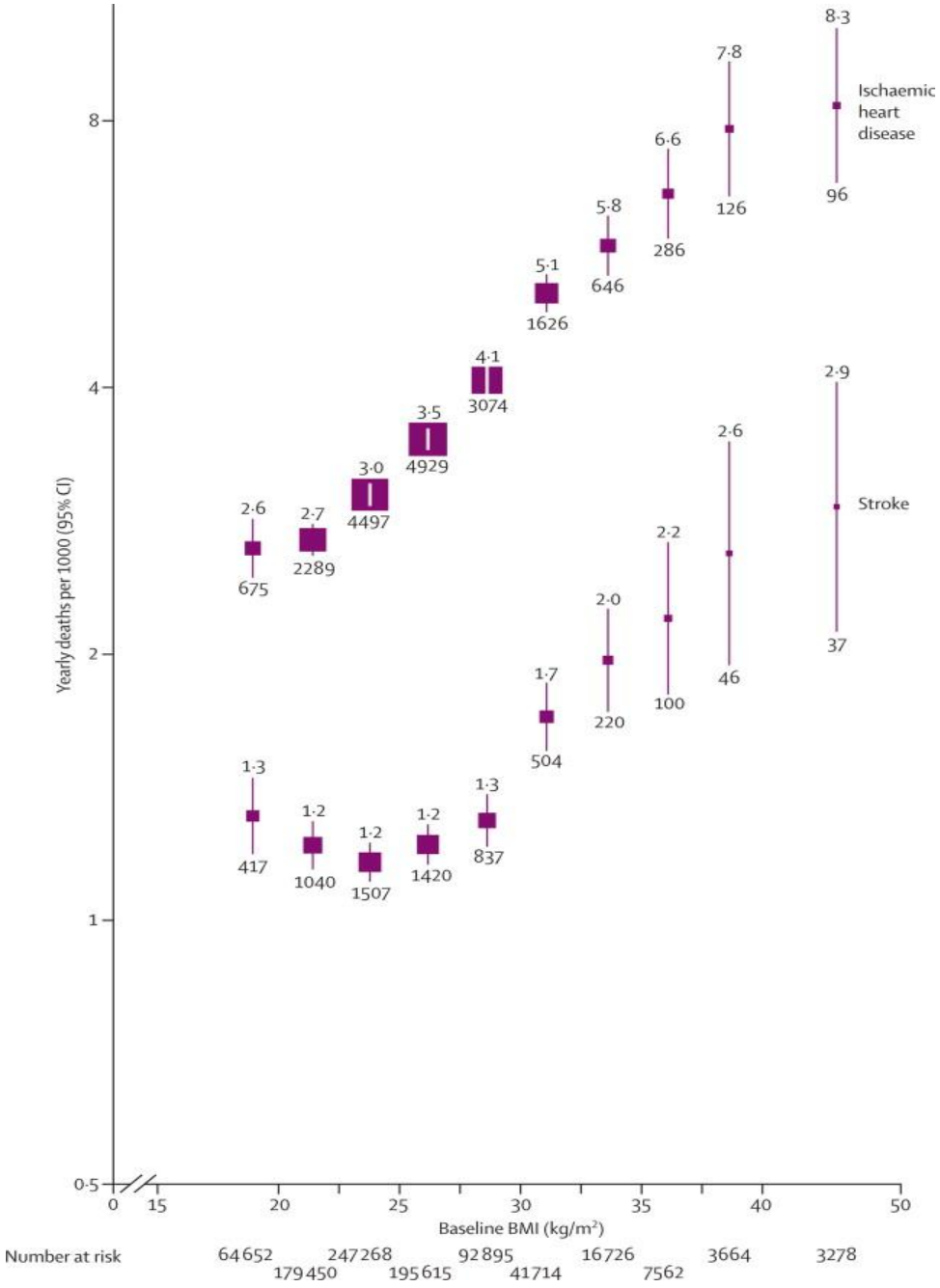


Figure 1.5 Relative risks with 95% confidence interval of ischaemic heart disease and stroke mortality versus BMI in the range 15–50 kg/m^2 (excluding the first 5 years of follow-up) in subjects aged 35–89 years, adjusted for age at risk, sex, smoking, and study Area of square is inversely proportional to the variance of the log risk. The numbers below represent the number of deaths and the number above represent rates/1000 population. From (Whitlock *et al*. 2009).

Risk of CVD increases markedly with age and is higher in men than in women. A family history of a parent or a sibling with CHD under the age of 55 years is strongly associated with increased risk. In a clinical setting various algorithms such as the Framingham Risk Score or the QRISK score (www.qrisk.org) are used to assess risk based on age, gender, BMI, systolic blood pressure and various measures of serum lipids (NCEP-3, 2001; Hippisley-Cox *et al.* 2007).

1.4.3 Plasma lipids

Generally, the risk of CHD is high in countries where the average serum cholesterol is high, compared to those where the average cholesterol concentration is low. Raised serum cholesterol concentrations are usually due to increases in the low density lipoprotein (LDL). LDL is derived from the metabolism of very low density lipoprotein particles (VLDL) that are secreted from the liver. VLDL is converted to LDL first by TAG removal from VLDL through the action of lipoprotein lipase to produce smaller particles, referred to as intermediate-density lipoproteins (IDL). IDL can be either cleared from the circulation by the liver particularly if they contain apolipoprotein E or converted to LDL by further removal of their TAG by hepatic lipase. LDL contains apolipoprotein B100 and is enriched with cholesterol. Serum LDL cholesterol (LDL-C) concentrations are usually estimated by determining the total cholesterol concentrations, high density lipoprotein cholesterol (HDL-C) and triacylglycerol (TAG) concentration using the Friedwald formula (total cholesterol – [HDL-C+TAG/2.2]). Desirable levels of LDL-C are less than 3 mmol/L. Serum LDL-C concentrations are determined mainly

by the activity of LDL receptors which are expressed mainly in the liver (Goldstein & Brown, 2009). Various hormonal and genetic factors influence the activity of the receptors. First discovered by Nobel Laureates Goldstein and Brown, this receptor was found to be lacking in patients with familial hypercholesterolemia (FH). Heterozygous FH, where only half of the numbers of LDL receptors are function, is characterised by very high LDL-C typically well above 6 mmol/L and a 25 fold increase in risk of CHD before the age of 60 years. The prevalence of FH is above 1:500 in the general population. Increases in LDL-C occur with increasing age and it is believed that LDL receptors activity declines with age and is affected by thyroid hormone activity and oestradiol. The increase in LDL-C seen in women post menopause is believed to be due to a decline in LDL receptors activity due to the loss of oestradiol. LDL particles play a crucial role in the atherogenic process, but native LDL particles required to undergo oxidative modification before they are taken up by scavenger receptors on macrophages within the sub-endothelial space (Steinberg, 1989). The accumulation of lipid laden macrophages results in fatty streaks in the artery and these can progress to form the more complex lesions of atherosclerosis (Ross *et al*. 1999). There is now firm evidence to show that lowering LDL-C by drugs slows the atherogenic process and reduces coronary events (Baigent *et al*. 2010). High levels of HDL-C, on the other hand, may exert a protective effect on the development of CVD by promoting the removal of lipid from fatty steaks. However, clinical trials have yet to demonstrate that raising HDL-C reduces risk of CVD and a Mendelian randomization study has failed to show an effect of HDL-C on risk of myocardial infarction (Voight *et al*. 2012).

1.4.4 Plasma triacylglycerols (TAG)

It has been shown that higher fasting serum TAG (>1.7 mmol/l) are associated with an increased risk of CHD. One mechanism involves unfavourable modifications to LDL and HDL particles. This happens through the transfer of large amounts of TAG to HDL and LDL particles, which is catalyzed by the cholesteryl ester transfer protein (CETP) reaction. After that, TAG in the HDL and LDL particles is hydrolyzed by the action of hepatic lipase resulting in the formation of small dense LDL and HDL particles. This leads to a rapid uptake of HDL particles by the liver and poor recognition of small LDL particles by LDL receptors. The consequences are a low level of HDL-C and high level of small dense LDL, which may be depleted in cholesterol content, but is indicated by increased apoprotein B (apoB) concentrations (Sarwar *et al*. 2010).

1.5 Effects of diet on plasma lipids

Obesity contributes to elevated LDL-C. Furthermore, central obesity is strongly associated with atherogenic dyslipidemia (hypertriglyceridemai and low HDL-C) and high proportions of atherogenic small dense LDL. Keys *et al.* (1957) and Hegsted *et al.* (1965) first observed that SFA C12-C16 raised TC when exchanged for other fats in experimental diets containing 40% energy from fat. Keys and Hegsted developed various equations to predict effects on TC. The most commonly used version is given below (Keys & Parlin, 1966).

Keys equation:

Δ cholesterol (mg/dL) = 2.3 ΔS –ΔP; which can be divided by 38.5 convert to mmol/L

ΔS is the difference in SFA % energy minus stearic acid and ΔP is the difference in energy % from PUFA.

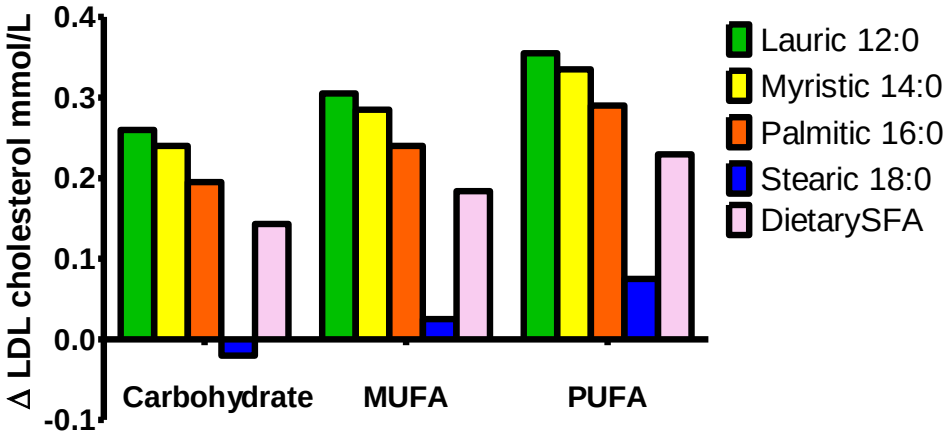
Numerous studies have subsequently evaluated the effects of different fatty acids on plasma lipid and lipoprotein concentrations and these have been subject to meta-analyses by Mensink *et al*. (2003).

SFA differ in their cholesterol-raising effects on the basis of the length of their carbon chain. Short (< 7) and medium chain (7-11) fatty acids have no effect on blood cholesterol because they are water-soluble and absorbed directly into the blood stream. Of the long chain fatty acids, stearic acid (18:0) has a negligible effect, possibly because it is desaturated to oleic acid (18:1*n*-9). Lauric (12:0), myristic (14:0) and palmitic acids (16:0), which are found in high proportions in dairy fats and tropical oils, increase LDL-C and HDL-C. In comparison to other SFA or unsaturated fatty acids, lauric acid was found to have the largest HDL-C raising effect and myristic acid raised LDL-C to the greatest effect (Mensink *et al*. 2003). The mechanism by which these fatty acids influence LDL-C is uncertain, but is probably due to an effect on LDL synthesis rather than catabolism via LDL receptors. The net effect of replacing these C12-C16 saturates with CHO on the LDL-C:HDL-C ratio or TC:HDL-C is neutral but the ratio falls when

they are replaced with MUFA or PUFA (Mensink *et al.* 2003). TFA increase both LDL-C and the LDL-C:HDL-C or TC:HDL-C ratio compared to CHO and thus has the worst net effect on the lipid profile. PUFA slightly lower the TC:HDL-C ratio more than MUFA. One possible mechanism for the effect of TFA is increase in the activity of cholesteryl ester transfer protein (CETP) (Abbey & Nestel, 1994).

PUFA, when consumed in large amounts act as ligands for the PPAR-alpha receptor which inhibits TAG synthesis, decreases VLDL production and promotes fat oxidation by the liver (Jump, 2011). LA appears to upregulate the expression of LDL receptor protein leading to rapid LDL removal from the circulation according to studies in hamsters and primates (Hayes & Khosla, 1992). In human studies, replacing SFA with PUFA increases the fractional catabolic rate of LDL (Goodnight *et al*. 1982) which would be consistent with increased removal of LDL by LDL receptors and increased bile secretion. Consumption of *n*-3 PUFA from fish oil markedly lowers TAG but does not lower LDL-C and may increase it in some individuals (Sanders, 2009a). At intakes of about 3g/d, it lowers VLDL TAG and synthesis, but lower intakes have a small effect. However, studies using purified DHA have found that it increases both LDL-C and HDL-C without any effect on TC:HDL-C ratio (Bernstein *et al.* 2012).

Panel A.



Panel B

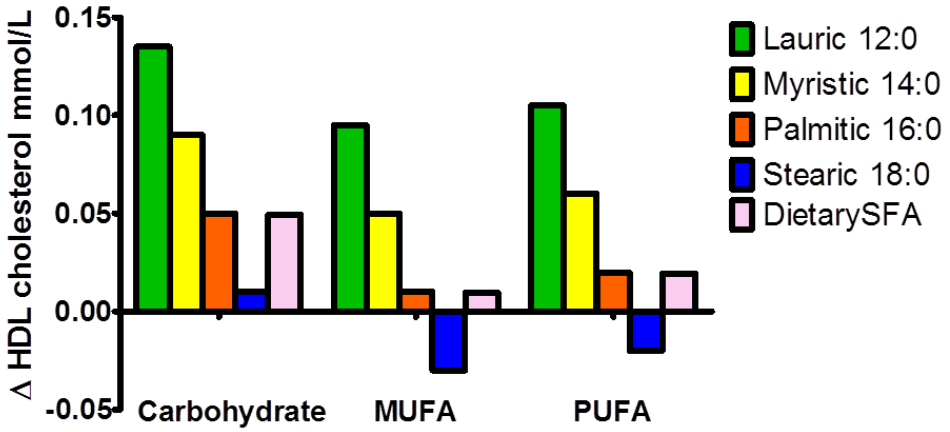


Figure 1.6 Predicted changes in low density lipoprotein cholesterol (LDL-C) (Panel A) and high density lipoprotein cholesterol (HDL-C) (Panel B) concentrations that result from exchanging 5 % energy as carbohydrate or monounsaturated (MUFA) or polyunsaturated (PUFA) fatty acids with different saturated fatty acids (SFA) or a mixture of dietary SFA, representing the proportions in the UK diet. Adapted from Mensink *et al*. (2003).

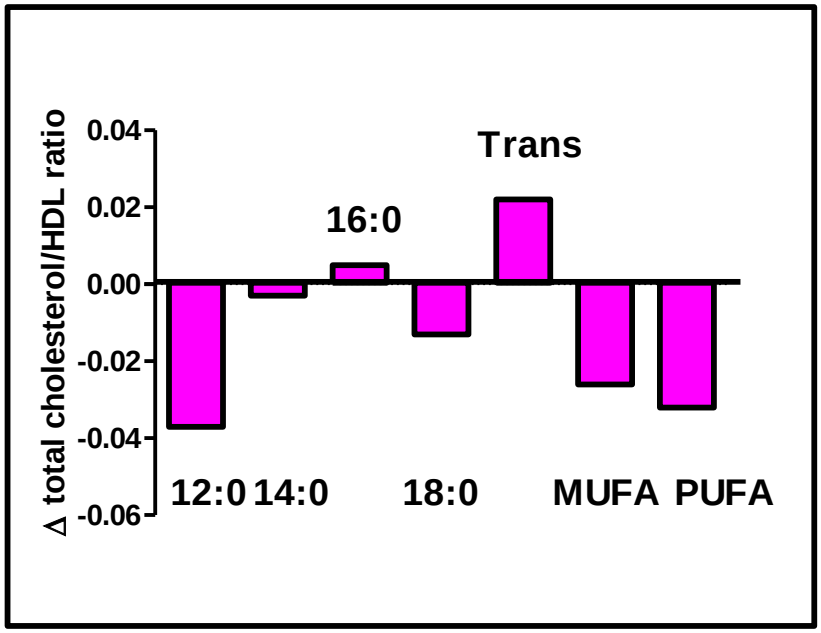


Figure 1.7 Effects (mean values with 95% CI) on the ratio of total cholesterol:HDL cholesterol (TC:HDL-C) resulting from the exchange of 1% energy as carbohydrate with equivalent amounts of food energy provided by different saturated, *trans*, monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids. Adapted from Mensink *et al.* (2003).

1.6 Dietary fat and risk of CVD

Keys proposed that the intake of SFA was strongly related to risk of CHD based on the Seven Countries Study, which was an ecological study (Keys *et al*. 1986). However, this simplistic assertion has been strongly challenged (Astrup *et al*. 2011) because it does not agree with recent systematic reviews of prospective cohort studies that show no relationship between the intake of SFA and CHD mortality (Skeaff & Miller, 2009; Siri-Terano *et al*. 2010). In contrast, a more consistent positive relationship between TFA intake and CHD mortality has been observed (WHO/FAO, 2010). It was estimated that replacement of TFA with SFA or MUFA would lower risk (Mozaffarian & Clarke, 2009)**.** Jakobsen *et al*. (2009) in a separate analysis examined the relationship between MUFA, PUFA and CHO intake on the risk of CHD from 11 American and European cohort studies. They found that a 5% energy replacement of SFA by PUFA leads to a significant reduction in risk from CHD mortality (hazard ratio: 0.87; 95% CI: 0.77, 0.97), while replacing SFA by CHO leads to a significant direct relationship (hazard ratio: 1.07; 95% CI: 1.01, 1.14). MUFA appeared to non-significantly increase risk compared with SFA but this finding may have been confounded by the inclusion of TFA with the MUFA. Hooper *et al*. (2012) in an updated Cochrane review of dietary interventions concluded that modification of fat intake, but not replacement of fat by carbohydrate, resulted in a significant 14% reduction in CHD incidence and a non-significant 7% reduction in CVD mortality. However, Hooper concluded that modification and reduction of fat intake might be even more favourable. In another

review, Hooper *et al.* (2012) concluded that dietary advice to decrease fat intake was associated with moderate weight loss, in this respect it would be predicted that a lower intake of fat might be associated with a lower risk as a reduction in body weight is associated with favourable changes in blood pressure and blood lipids.

1.7 Dietary reference intakes for fat and fatty acids

Several bodies such as WHO/FAO, EFSA and the Institute of Medicine (IOM) in the USA have recently reviewed recommendations for fat intake, which are summarised in **Table 1.3**. Current UK guidelines are based on the COMA report (Department of Health, 1994) and are incorporated into NICE Guidelines (CG 67; www.nice.org.uk). In the UK for example, the contribution from fat should not exceed 35% of energy, no more than 10% of energy should come from SFA and less than 2% from TFA. The UK has a dietary reference value (DRV) of 6% energy from PUFA, but makes no specific recommendations for MUFA. In most guidelines, estimates of acceptable intakes for MUFA are based on the calculations by difference (Department of Health, 1994). The WHO/FAO 2009 consultation (WHO/FAO, 2010) suggested average macronutrient distribution ranges for *n*-6 and *n*-3 PUFA combined as 6-11.5% energy. The slightly higher value for the upper limit was due to considerations regarding *n*-3 fatty acids (WHO/FAO 2010). All three recent reports now have total fat intakes in the range of 20-35% energy. There is also general agreement with an upper limit of 10% for SFA

but EFSA has specified as low as possible. More recent (**Table 1.3**) recommendations for TFA are no more than 1% energy or as low as possible. Neither EFSA nor IOM specified a DRV for PUFA. The ranges of acceptable intakes for PUFA in the IOM report were similar to the WHO/FAO whereas the acceptable intake was lower in the EFSA report. In the UK, the National Diet and Nutrition Survey data (NDNS) show that total fat intake and PUFA is close to the UK recommendations but the intake of SFA is above the target (Henderson *et al*. 2003). The same pattern is seen in most European countries and in North America and Australia. Generally, over the past thirty years intakes of total fat expressed as a percentage of the energy have fallen. This may seem surprising in the light of the major increases in vegetable oil production. There is, however, a possibility that actual intakes of fat may be substantially under-reported and that there may be a mismatch between estimates of fat moving into consumption from actual reported intake. The various techniques for measuring dietary fat and their limitations are discussed in the following section.

Table 1.3. Summary of dietary recommendations for the intakes of total fat and fatty acids intakes for adults as set by different organizations.

	Fat	SFA	MUFA	PUFA	*Trans*	*n*-6	*n*-3	*n*-3 LCP
UK DoH 1994	<35%	DRV <11%	NA	DRV 6%	<2%	EAR 1%	EAR 0.2%	DRV 0.45
FAO/WHO 2009	20-35%*	<10%	NA	6-11.5*	<1%	2.5 -9.0%	0.5-2.0%	0.25g-2g
EFSA 2010	20-35%	ALAP	NA	No DRV	ALAP	AI 4.0%	AI 0.5%	0.25g
Dietary guidelines for Americans 2010	20-35%*	<10%	NA	No DRV	ALAP	AI 5-10% *	AI 0.6-1.2%*	NA

DRV: dietary reference value; EAR: estimated average requirement; AI: acceptable intake; ALAP: as low as possible; NA:not advised.

* Average macronutrient distribution range.

1.8 Assessment of dietary intake of fat

There is a great interest in understanding the relationship between fat intake and the development of chronic diseases including CVD and cancer, which leads to a pressing need for an accurate and precise measurement method that is able to reflect actual fat and fatty acids intake of an individual. Thus, it is very important to assess dietary fat intake using methods of measurement that are able to estimate the actual intake of type and amount of fat (Hodson *et al*. 2008). Methods used to obtain information on food and dietary intakes are either based on the intakes of groups of people or individuals.

1.8.1 Population studies

Information on food supply at the national level can be obtained using food balance sheets or food disappearance data, which provide a picture of per capita accessibility of food items. Thus, the figures offer an indication of food availability rather than intake or consumption. According to population studies, patterns of dietary intake can be categorized chiefly by the food sources of macronutrients, as countries differ in the accessible sort of CHO staples (wheat, potatoes or rice), proteins (animal, vegetable or fish) or fats (animal fat, tropical oils, coconut oil, palm kernel oils and others). Such data does consider non-food uses of fat (soap, biodiesel) or exports. For example, a food balance sheet approach would indicate that Malaysia and Indonesia have high per capita availability of fat but most is exported and fat intakes are in the range of 25-30% energy (Thomson & Subar, 2001; Eastwood, 2009).

1.8.2 Community data

Community data based on surveys of groups of people rather than individuals and can be utilized for community surveys of nutrients intake and for identifying different food consumption patterns. It provides community, socioeconomic and geographic information and permits comparison between different times and regions. Food accounts, inventories, household record and list recall are the four major methods of measurements used in community surveys. This method becomes unreliable when large amounts of food are eaten outside the home and it gives no indication of distribution of food intake within households (Thomson & Subar, 2001; Eastwood, 2009).

1.8.2.1 Food accounts

This method is based on the household where the person liable for purchasing and preparing the food maintains a record of the amounts of food items entering the house. These comprise purchases, garden food, gifts, payment in kind and others. This kind of survey relies on the assumption that the average level of food stocks within the houses food store is stable, which is highly unlikely. In Great Britain, the Household Food Consumption and Expenditure Survey (MAFF, 1991) started in 1940 to evaluate the nutritional quality of the diet of urban, working-class households, to track the significance of the wartime food rationing policy, which finished in 1954, but continued until 2000. Thus, these reports provide a broad account of the British food habits since then i.e. over 60 years. The survey became unreliable when the proportion of food purchased outside the home increased (Thomson & Subar, 2001; Eastwood, 2009).

1.8.2.2 Inventories

Using the inventory method, respondents are asked to keep records of individual food items entering the house. At the beginning and end of the survey, a larder inventory is accomplished. This method allows for direct measurement of the quantity of food and nutrients available within a single household (Thomson & Subar, 2001; Eastwood, 2009).

1.8.2.3 Household record

In the household records, household measures are used to weigh or estimate the foods available for consumption (raw or processed) allowing for preparation waste. Then food eaten is calculated and subtracted from the total. This technique is ideal for population consuming home based diet and for simple and unsophisticated societies. In some communities measurements of food consumption of individuals is sophisticated and requires careful studies that begins from meal preparation and continue to watching the individuals eat (Thomson & Subar, 2001; Eastwood, 2009).

1.8.2.4 List recall

In this method, the individuals are requested to recall the amount and cost of food acquired for household use over a given period usually from 24h up to a week. This technique helps to estimate food cost and net household consumption of foods and nutrients, and is ideal when most of the consumed food is purchased rather than home produced (Thomson & Subar, 2001; Eastwood, 2009).

1.8.3 Individual data

1.8.3.1 Description, weighed and estimated records

In this method each item consumed has to be weighed before consumption by either participants themselves or an investigator in addition to recording a detailed description of the consumed food in a specially designed booklet. Participants can be taught by trained personnel to describe and provide detailed information of the weighed food prior to consumption and record any leftover. Weighed intake can be measured for up to three, four, five or seven days. A 7-day weighed intake is widely used and has the advantage of providing a more precise estimation of portion size. High respondent burden, misreporting, and the cost of the method are among the main limitations associated with this method. Because of the high subject burden and the time spend for coding food; this method has often been not used in epidemiological studies (Wrieden *et al.* 2003; Thomson & Subar, 2001; Eastwood, 2009).

1.8.3.2 Diet histories

Diet histories collect information on frequency and quantity of food consumed on previous occasions and accuracy of these methods rely mainly on memory. The optimal period for collection data on dietary intake is 3-4 days. However longer periods are necessary for alcohol, several vitamins, minerals and cholesterol. Only 24h recalls will be addressed as they are widely used. It is crucial that a diet history consist of:

A detailed interview to determine quantity and frequency of food

A cross-check frequency list

A 24h recall

A 3-day record (voluntary)

The main disadvantages of diet histories include dependence on memory, interviewer bias and participant bias as a result of being interviewed. Furthermore, these methods are of limited value in some population groups such as children under the age of 12 years. On the other hand, retrospective methods are quick, cheap, and require no motivation and literacy (Eastwood, 2009).

1.8.3.3 24h recall

24h recall, like other diet histories, relies on memory to recall intake in the recent past days. Subjects are requested to recall and describe all food and drinks consumed in the 24h before the interview by asking a systematic series of questions. The focus of the questions is on amount and frequency of food intake meal-by-meal and looking for day-to-day and seasonal differences. The accuracy of this method is highly dependent on the ability of the subjects not to under or misreport their intake and to estimate the portion size precisely. This can be helped by using a well-trained interviewer who can assist in the process of recalling and estimation of portion size. This method causes low burden on the subject and can be administered by the use of telephone and thus is suitable for large-scale surveys. The main limitation is that this method is not representative of usual intake due to variations in food intake from day to day and differences between

the weekday and weekend intake. In addition, this method, apart from being memory dependent, is likely to be associated with bias in relation to estimation of portion size and in recording the "good/bad" food. For more accurate data a multiple 24hr recalls might be applied, which may add extra burden on the subject (Nelson & Bingham, 1997). Moreover, peoples with low intake are liable to report higher than actual intake, and those with higher intake are likely to report lower (Thomson & Subar, 2001; Eastwood, 2009).

1.8.3.4 Questionnaires

Questionnaires are one of the most widely used tools for assessment of dietary intake. Subjects are asked about the frequency of consumption of food or drink. They are easy, cheap and can be self or interviewer administered. When self-administered, interviewer bias is eliminated. However, questions have to be very straightforward and explicit or they may be left unanswered. Questions are closed or open, simple or comprehensive, and may comprise from 9 to 190 food items (Thomson & Subar, 2001; Eastwood, 2009).

1.8.3.5 Food frequency questionnaire

Food frequency questionnaires (FFQs) attempt to collect information about food consumption from a large number of individuals (100 or more) beside information regarding portion size and incidence of consumption within a specific period and therefore ability to reflect habitual intake. It consists of a list of food coupled with a

selection of options regarding frequency of consumption of each food in the list. The length of the food list varies depending on the nutrient under investigation and its sources. Because the FFQ has a low respondent burden and can be self-completed, it is suitable for use in a large epidemiological studies and surveys. However, problems such as portion size estimation, over-reporting of healthy foods (e.g. fruits and vegetables and dairy products) and under-reporting of unhealthy food raises questions regarding their ability to provide an accurate assessment of fat intake. Particularly when the FFQ is unable to discriminate, which fats and oils are used in compound foods and a number of assumptions are made regarding portion sizes, which in the case of fat, which is energy dense, could lead to large errors. For instance, Bingham *et al*. (2003) investigated the relationship between SFA intake and breast cancer in 13070 women between 1993 and 1997 showed that the risk of breast cancer was associated with SFA intake when assessed by the 7-day food diary (P=0.005) but was unable to demonstrate the relationship when FFQ (P=0.23) was used.

1.8.4 Limitations and sources of errors

Table 1.4 summarises the limitations of the various methods to assess fat intake. Most epidemiological studies have used FFQs to estimate dietary intake of fat and fatty acids and relatively few have used weighed food records that are the most reliable. More recent surveys in the UK have used repeat 24h recalls or 4-d record. However, a major problem with these methods is the assessment of portion size and potential for recall bias.

Table 1.4 Summary of limitations of dietary assessment methods with regard to type of fat

Method	Subject burden	Mis-reporting	Miscoding	Accuracy	Uncertainty
Duplicate diets	Very High	Low	N.A.	High	Moderate
Weighed intake	High	Moderate	Moderate	Moderate	Moderate
FFQ	Low	Moderate	High	Low	Low
24h recall	Low	High	Moderate	Low	Low
7-day food diary	High	Moderate	Low	Moderate	Moderate

Mis-reporting – both over and under-reporting

Miscoding – difficulties in assessing correct food code or portion size

Accuracy - problems with recall, description of foods consumed

Uncertainty – participants may change what they eat to make recording simpler

In comparison to other macronutrients in the diet, amount and type of dietary fat is one of the most difficult to measure using traditional assessment methods due to a number of reasons. Added fat used in food preparation including frying, cooking and dressing, is very difficult to recognize and quantify. Furthermore, bias is associated with accuracy of reporting foods high in fat, the problem that is well known among overweight and obese persons, who tend to underreport their intake (Arab, 2003). Fat can be subject of recall bias. Traditional assessment methods, such as FFQ and 24h recall, which are the main assessment methods used in epidemiological studies to estimate individuals and populations habitual intake, are subject to limitations and measurement errors that are associated with recall and data collection and processing. These limitations include

systematic bias, interviewer bias, social desirability bias and miscoding in the food composition table (Nelson & Bingham, 1997).

1.9 Current intakes and sources of fat in the UK diet

In terms of sources of fat intake, NDNS has provided useful data. The earlier surveys are probably more robust as they recorded weighed food intakes, whereas the more recent surveys resorted to using multiple pass food recalls. Using the more robust NDNS data (Henderson *et al.* 2003), the main sources of fat in the UK diet were meat and meat products (23%), cereal and cereal products (19%, this included added fats in processing), milk and milk products (14%), fat spreads (12%) and potatoes and savoury snacks (10%) as shown in **Figure 1.8**. The contribution of SFA to the energy intake was similar at 13.4% and 13.2% for men and women respectively. The mean percentage of food energy from TFA for both men and women was 1.2% lower than the old target but still above 1%. Regarding the *cis* MUFA, the average intakes were 12.1% and 11.5% for men and women respectively. For the *n-3* PUFA, the mean intake was greater in men than women and was lower for women aged 25 to 34 years than those between 50 to 64 years of age. There were no reliable estimates of short-chain SFA and LC *n-3* PUFA. The average daily intake for *n*-6 PUFA was significantly higher in men than women ($P<0.01$) but based on percentage energy they were similar (Henderson *et al.* 2003). Most of the SFA were derived from meat and meat products and milk and milk

products, although the contribution from milk products has fallen markedly since earlier surveys. Reliable data on changes in the fatty acid intake of the UK and other European diets are hard to assess, and the food industry is continually modifying the composition of fats and oils to meet consumer demands as well as keep ingredient cost as low as possible. The UK food composition data base is limited and much of the data are based on the analyses having been conducted over 20 years ago (Holland *et al.* 1991; MAFF 1998) and the published food tables lack information on the detailed fatty acid content of commonly consumed foods being restricted to total fat, SFA, MUFA, *trans* isomers and PUFA. There is a pan-European project called EUROFIR (www.eurofir.org) which is trying to address issues of food composition in the European Union. The situation with regard to accurate food composition data is even more vague in many emerging economies particularly those of the Middle East where there is only limited food composition data available.

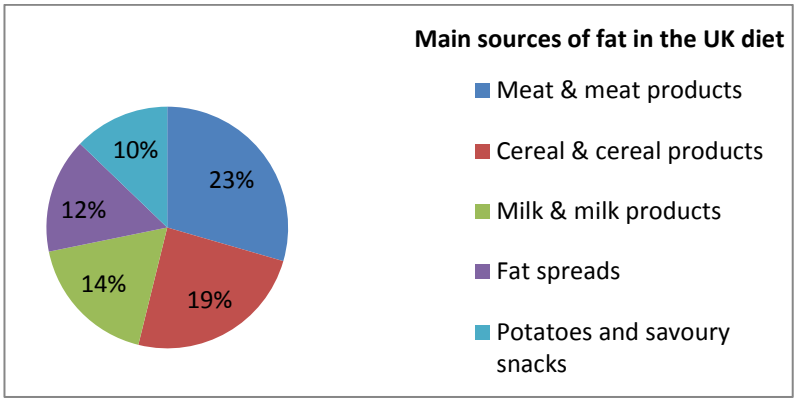


Figure 1.8 Pie chart showing main sources of fat in the British diet (Henderson *et al.* 2003)

1.10 The use of biomarkers for assessment of fat intake

1.10.1 Major types of biomarkers used in estimating fat intake

1.10.1.1 Adipose tissue

Adipose tissue is the main fat storage site. It has been considered as a suitable representative of long-term dietary intake of fat because it has a slow turnover time in weight stable subjects, which is about 1.5 years (Strawford *et al*. 2004). In healthy adults, adipose tissue consists approximately of 99% TAG, 0.3% cholesterol, and less than 0.1% phospholipid (PL) (Hodson *et al*. 2008). The major fatty acids in adipose tissue are oleic acid (18:1*n*-9; 37.0-49.7 mol%), palmitic acid (16:0; 18.1-23.5 mol%), LA (18:2*n-6*; 8.6-24.9 mol%), palmitoleic acid (16:1*n*-7; 4.5-10.1 mol%), stearic acid (18:0; 2.6-4.7 mol%) and a very low proportion of very LC-PUFA (<1 mol%). There are some difference in composition between different adipose tissue sites with high proportions of MUFA in subcutaneous fat and lower proportion of SFA compared with abdominal fat, especially perirenal fat, but they lack any differences in the distribution of LA (Hodson *et al*. 2008).

Six studies, investigated the composition of human adipose tissue as reliable biomarker of dietary fat intake, were found. All studies analyzed fatty acid composition of human tissues by gas chromatography (GC) and dietary assessment by FFQ. Among these, one study measured habitual fish intake by analyzing marine LC *n*-3 PUFA composition of adipose tissue only (Marckmann *et al*. 1995); one study measured TFA only (Clifton *et*

al. 2004); two studies measured ALA and the risk of nonfatal acute myocardial infarction (Baylin *et al*. 2003; Campos *et al*. 2008). Two further studies were conducted to investigate the reliability of adipose tissue as biomarker for a range of fatty acids (Baylin *et al*. 2002; Craig *et al*. 2009).

All six studies investigating human adipose tissue indicated that its composition reflects that of dietary fat intake as assessed by FFQ. It has been shown in case-control studies on Australian cohorts that adipose tissue TFA composition is significantly associated with the intake of TFA from the diet (18:1*n*-9, r=0.66, 18:1*n*-10, r=0.66) (Clifton *et al*. 2004). Another interesting point is that when TFA were removed from the margarine in mid-1996, levels of TFA (18:1*n*-9 and 18:1*n*-11) were higher in cases biopsied before mid-1996 ($P< 0.03$, $P< 0.001$ respectively). This study concluded that TFA in the diet correlated with adipose tissue TFA (18:1*n*-9 and 18:1*n*-10) indicating that adipose tissue is a good biomarker of dietary TFA intake (Clifton *et al*. 2004). An initial study in Costa Rica found lower levels of ALA in the adipose tissue of patients who had suffered a myocardial infarction (MI) compared with their controls (Baylin *et al*. 2003). In a follow-up of this study, Campos *et al*. (2008) investigated the intake of ALA and the risk of MI on 1819 cases and equivalent number of controls and showed that estimates of dietary ALA intake were correlated with the proportion of ALA in adipose tissue. When using adipose tissue as biomarker of habitual fish intake, the results showed that the consumption of fish (r= 0.55) and marine *n*-3 PUFAs (r= 0.58) were significantly associated with DHA content of adipose tissue ($P< 0.001$), but were

not significantly associated with EPA and DPA (Marckmann, 1995). The remaining two studies compared the reliability of the use of adipose tissue as a biomarker of different fatty acids. The first one concluded that adipose tissue is suitable for the assessment of *n*-3 (r=0.24), *n-6* (r=0.58) PUFA and TFA (r=0.58) intake from the diet, (Baylin *et al*. 2002). The other study reported the reliability of adipose tissue to significantly reflect DHA (*P*=0.037) and EPA (*P*=0.018) (Craig *et al*. 2009).

In conclusion, the evidence suggests that adipose tissue can be utilized to estimate intake of PUFA and TFA. The reliability for assessing intake of MUFA and specific SFA remain uncertain.

1.10.1.2 Plasma total lipids

Plasma total fatty acid composition typically reflects a mixture of all plasma lipid fractions that contain fatty acid in their structure. These include PL, TAG, cholesteryl ester (CE) and non-esterified fatty acids (NEFA). The fatty acid composition of plasma total fatty acids is in the following order 18:2*n*-6 (30.4 mol%), 16:0 (23 mol%) and 18:1*n*-9 (19.5 mol%). These lipid fractions have different fatty acid composition and thus any change in the concentration of any of these lipid fraction results, in turn, in a distinctive difference in plasma total fatty acids composition. Typically, the concentration of PL is about 1 mmol/L, NEFA 0.6 mmol/L, CE 4 mmol/L and TAG 1 mmol/L. So the molar contributions of fatty acid from PL, NEFA, CE and TAG can be estimated as 2, 0.6, 4 and 3 respectively. Hodson *et al*. (2008) ranked fatty acids in plasma according to how much they varied between subject in the following order

20:4*n*-6, 20:5*n*-3, 22:6*n*-3, 18:1*n*-9, 18:2*n*-6, 16:1*n*-7, 18:0 and 16:0. Palmitic and stearic acids were surprisingly the least variable given the relatively large range of variation in SFA intake between individuals. Plasma CE are particularly enriched in LA probably because it derives its fatty acids from lecithin via the enzyme lecithin cholesterol acyl transferase (LCAT) and lecithin is rich in LA. NEFA in the fasting state tend to represent the fatty acid composition of adipose tissue whereas in the postprandial state they represent the composition of the dietary fat ingested. TAG in the fasting state represents TAG synthesised in the liver, which is dependent on recent dietary intake as well as the flux of NEFA from adipose tissue. Following a fatty meal, the TAG composition will tend to be more similar to that of the fat consumed. Some researchers have measured total phospholipids or lecithin as biomarkers of intake but total plasma fatty acids appear at least a robust index of fatty acid intake and are much easier to measure directly than PL (Lepage & Roy,1986) and is widely used (Kuratko & Salem, 2009).

1.10.1.3 Plasma phospholipids

Plasma PL composed of a glycerol molecule having two fatty acids at positions *sn*-1, which is predominated by SFA or MUFA, and *sn*-2, which is predominated by MUFA or PUFA, and a phosphoric acid residue at the third position. The PL species include sphingomyelin (SPH), lecithin/phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylethanolamine (PE). Lecithin and SPH are the main fractions in plasma. SPH is not sensitive to changes in dietary fat but PC is very

sensitive to the level and type of PUFA. The major fatty acids of plasma total PC are in the order of 16:0, 18:2*n*-6, 18:0, 18:1*n*-9, 20:4*n*-6 and 22:6*n*-3 (Sanders *et al*. 1978; Hodson *et al*. 2008).

1.10.1.4 Erythrocyte phospholipids

Erythrocyte phospholipids are characterized by a large amount of 16:0 (21.2-34.9%), which is twice as abundant as any other fatty acid. The proportions of 18:0, 18:1*n*-9 and 20:4*n*-6 were around 14%. 18:2*n*-6 has a lower quantity (11.2%). With reference to plasma PL fatty acid composition, the profusion of very LC *n*-3 PUFA is equivalent to that of plasma PL. However, the abundances of 18:2*n*-6 and 20:4*n*-6 are half and double that of plasma PL respectively. This lower amount of 18:2*n*-6 in erythrocyte PL is a typical feature of cell membrane and indicates a tight metabolic regulation of the composition of this fatty acid (Hodson *et al*. 2008). Erythrocyte lipids have been widely used to assess the balance between *n*-6 and *n*-3 fatty acids in the diet but are relatively insensitive to changes in SFA intake. Erythrocyte phospholipids are considered a longer-term indicator of past intake. Although erythrocytes have a life span of about 120 days, it is possible to bring about changes in their composition within a few days and while changes in the proportion of LC *n*-3 PUFA persist for longer following withdrawal of supplementation, values for EPA still changed over a 5-week period (Sanders *et al*.1981).

1.10.1.5 Other tissues i.e. buccal cells

The use of buccal cells as biomarker of fatty acids intake has been used as a reliable marker of LC-PUFA status as the PL are the major component of cell membranes. Buccal cells are preferred over other biomarkers when measuring fatty acids composition in infants. This is because this tissue is available and helps avoiding repetitive blood sampling. In addition, small amount of oral mucosal cells are sufficient for the analysis and detectable by the GC. Furthermore, this fraction has a rapid turnover rate, which is 5 days, and thus reflects current intake and is sensitive to changes in dietary intake. Moreover, this tissue is not affected to a great extent by contamination with dietary lipid, which is predominantly composed of TAG. The main SFA in cheek cells are palmitic and stearic acids. Oleic acid has the highest proportion among the unsaturated fatty acids. For the LC-PUFA, the order of the fatty acids is, from highest to lowest, 18:2*n*-6, 20:4*n*-6, 22:6*n*-3, 20:3*n*-6, 20:5*n*-3, 20:2*n*-6, 18:3*n*-3 and 18:3*n*-6. In conclusion, cheek-cell lipid analysis is of possible value in nutrition research studies and of useful use in epidemiological studies on fatty acid analysis particularly from children (Laitinen *et al*. 2006; Koletzko *et al*. 1999; McMurchie, 1984). However, the amount of material for analysis is very small and there is the danger of contamination of swabs with food particles.

1.10.2 Previous studies using biomarkers of fat intake

Four studies were identified from a Medline search. One was on ten European countries (Denmark, France, Greece, Germany, Italy, the Netherlands, Norway, Spain, Sweden,

and the United Kingdom) contributed in the EPIC study. This was a cross-sectional study to determine the plasma fatty acids composition of 3,000 subjects or more from 16 different European countries. The authors aimed to analyze the relationship between the plasma PL fatty acid concentration and dietary intake of major food groups using FFQ and a single 24hr recall. They examined the potential of two different dietary assessment methods to predict plasma fatty acids concentration at a population level. In addition, they tried to evaluate the influence of the endogenous (age, sex and BMI) as well as the exogenous (region and lab variables) factors on plasma fatty acid concentration as a secondary objective. A total of 22 fatty acids were separated from fatty acids methyl ester (FAME) of chain length between 14 and 22 carbon atoms and of different fatty acid classes including SFA, MUFA, PUFA, and TFA using GC technique. This study showed that specific plasma PL fatty acids are considered good biomarkers of some, but not all, types of food consumed in each region of the EPIC study, which suggest differences in food intake between regions under study. For example, some patterns of fatty acids appeared to be linked to specific dietary groups. For instance there was a significant correlation with mean high intake of fish and LC *n*-3 PUFAs (r=0.78, $P<0.01$), olive oil and oleic acid, *cis* 18:1*n*-9, (r=0.73, $P<0.01$) and margarine and elaidic acid, TFA 18:1*n*-9, (r=0.76, $P<0.01$). The observed association was strongly determined by region, which implies that there is variation in dietary intake across regions. For example, olive oil intake was correlated with oleic acid (*cis* 18:1*n*-9) concentration only in areas where consumption of olive oil was high, but the association was absent in those centers of subjects with low intake. This study confirms

that plasma PL concentration can be used as a reliable biomarker of some fatty acids, especially those that are not endogenously synthesized such as branched chain fatty acids and TFA (Saadatian-Elahi *et al*. 2009).

Chajes *et al*. (2008) investigated the relationship between fatty acid composition of serum PL and the risk of breast cancer among women in the French component of the EPIC study. A total of 19,934 women, who has been followed up for seven years, were matched by controls for age, menopausal and fasting status at the time of blood sampling, and date and center where collection took place. This study showed that a high level of serum *trans* MUFA (palmitoleic and elaidic acids) was associated with increased risk of breast cancer. Dietary consumption of foods rich in *trans* MUFA assessed by FFQ significantly correlated with serum PL levels of these fatty acids (*trans*-palmitoleic, *P*=0.003 and elaidic acids, *P*=0.0001) (Chajes *et al.* 2008).

A further case-control study aimed to evaluate the relationship between levels of different TFA of plasma PL in older adults and the development of fatal Ischemic Heart Disease (IHD) and sudden cardiac death (Lemaitre *et al*. 2006). They found that elevated levels of TFA 18:2 (OR 2.34, 95% CI 1.27-4.31) and decreased levels of TFA 18:1 (OR 0.18, 95% 0.06-0.54) are associated with an increased risk of fatal IHD and sudden cardiac death, (Lemaitre *et al*. 2006). The last study examined the association between plasma PL and CE fatty acid composition and those of habitual diet measured by FFQ in 3570 free-living middle-aged adults. The results of this study showed that dietary SFA was moderately correlated with plasma PL and CE (r=0.15 and r= 0.23

respectively), MUFA was weakly correlated (r= 0.05 and r= 0.01 respectively) and PUFA was well correlated with both plasma PL (r= 0.25) and CE (r= 0.31). Of all PUFA, plasma DHA was strongly correlated with dietary intake (r= 0.42). Moreover, they found that dietary SFA was positively associated with concentration of plasma MUFA and 18:1*n*-9 (both r= 0.26). On the contrary, MUFA and 18:1*n*-9 from the diet were not associated with plasma concentrations of MUFA and 18:1*n*-9 (range r= -0.02-0.06). Whereas, plasma concentrations of EPA and DHA were associated with dietary intake of LA (r= 0.15) and, interestingly, the strongest correlations were observed between plasma and dietary intake of both EPA (r= 0.44) and DHA (r= 0.42). SFAs and LA were chosen to perform a further analysis to examine the effects of disease status, body fatness, smoking and alcohol drinking. The statistical analysis revealed that male participants with chronic disease (i.e. having either CVD, diabetes or hypertension), who were overweight, smokers, or who drank alcohol heavily, had an elevated level of mean plasma SFA and/or decreased levels of mean plasma LA than those who were free from these factors (Ma *et al.* 1995).

Two studies have compared both subcutaneous adipose tissue biopsies from the upper buttock and plasma as biomarkers of fat intake. The first one examined the hypothesis that high ALA in plasma and adipose tissue is associated with reduced prevalence of the metabolic syndrome (MS). They found that elevated levels of ALA were associated with a lower risk ($P< 0.02$) (Truong *et al.* 2009). The purpose of the last study was to examine the reliability of the fasting whole blood in reflecting dietary intake of fat as

compared to adipose tissue and plasma in 101 females and 99 males in Costa Rica between 1999 and 2001. All participants completed 135-items FFQ and supplied adipose tissue in addition to fasting blood samples. Fatty acid analysis of the samples of blood and adipose tissue were analyzed by gas chromatography. The authors found that strongest correlation with dietary intake was for adipose tissue with the highest correlation for ALA (P= 0.024), LA (P= 0.039), and 18:2 *trans* as compared to plasma. The authors concluded that fasting whole blood is a suitable biomarker for long-term LA and ALA intake. Fasting whole blood require minimum sample processing, accessible as well as its ability to predict dietary intake as good as fasting plasma (Baylin *et al.* 2005).

The majority of the studies included in this review were consistent in supporting the hypothesis that adipose tissue and plasma fatty acid composition are valid biomarkers of PUFA, TFA and other exogenous fat (such as branched chain fatty acids) and should be used in line with FFQ. However, some studies showed that plasma can be used as biomarker for some (e.g. *n*-3 PUFA of marine origin), but not all (e.g. MUFA), fatty acids (Ma *et al*. 1995). Furthermore, obtaining adipose tissue biopsies is less acceptable for participants than giving blood and for large scale epidemiological studies. This suggests that plasma is the most suitable material for biomarker use. This is evaluated further in section 1.10.4.

1.10.3 Factors influencing fatty acid measurements in biomarkers

Measurement of fatty acids in biomarkers is influenced by a number of factors that may affect the actual levels of fatty acid in the analysed specimen. The first factor is handling and storage of the sample. For instance, oxidation of PUFA by exposure to air or contact with iron present in erythrocyte can happen because of inappropriate handling and storage. Long storage can result in sample degeneration or the consequences of refrigeration loss. In addition, changes in the PUFA profile can happen as a result of sensitivity to temperature and oxygen, which can be minimized by careful storage at -80°C under nitrogen gas or with added anti-oxidants such as butylated hydroxytoluene. Another important factor is tissue-sampling site. A comparison of fatty acid profile of deep seated sites (perirenal) and subcutaneous sites (abdominal and buttocks) show that perirenal adipose tissue has high proportions of SFA, while buttock has the lowest proportion of SFA and a highest proportion of MUFA. Abdominal adipose tissue consists mainly of SFA. PUFA profile showed no significant differences across the sites (Arab, 2003). Moreover, nutritional status of the subject can affect fatty acid profile of the individual. For example, adequate amounts of minerals including iron, zinc, copper and magnesium is required for the desaturase enzymes, which are metalloenzymes, to function properly. Furthermore, because the measurement reflects the percentage of individual fatty acids not the absolute amount of the consumed fatty acid, the increase in the intake of some fatty acids can drive down the relative percentage of the other even though its intake remains stable. Weight stable subjects are preferred to measure

accurately long-term intake. Changes in the diet of a normal weight person require three to four years for adipose tissue to equilibrate to the new diet. Adipose tissue have a tendency to reflect long-term dietary intake (~1-2y), which makes it impossible to obtain accurate dietary information from the respondent that reflect the same period of measurement. Consumed fatty acids in the diet are not stored directly, but some are oxidised while others undergo metabolic reactions before storage (Arab, 2003; Hodson *et al*. 2008).

1.10.4 Biomarkers of fat intake: A review of the cohort studies and trials

1.10.4.1 Background

In order to identify systematically cohort studies that have compared these biomarkers with estimates of dietary intake and randomized controlled trials that have validated biomarkers, a structured search strategy was adopted.

1.10.4.2 Review questions

The research questions posed were:

How well do biomarkers of fatty acid intake correlate with traditional estimates from dietary intake?

What evidence is there from randomized controlled trials of modification of fat intake to indicate that biomarkers of fatty acid intake are reliable indices of dietary intake?

1.10.4.3 Defining inclusion/exclusion criteria

Cohort studies were included in the review according to the following criteria:

There were at least 64 participants in the study.

Analysis of fatty acid composition of the biomarker by gas chromatography.

Dietary intake of fat is reported.

A statistical measure of association (Pearson's or Spearman's correlation coefficient) between proportions of fatty acids in biomarker and diet is reported.

Only English language publications were considered.

Criteria for randomized controlled trials are:

Participants were randomly allocated to control or treatment.

The sample size for each group was greater than 64 and the duration of intervention was at least 12 weeks.

Analysis of fatty acid composition of the biomarker by gas chromatography.

The composition of the dietary fat intake of the dietary interventions was reported.

1.10.4.4 Search strategy

Searches of electronic databases Embase, Scopus, Ovid Medline, Pubmed, Web of knowledge and Web of science using the following search terms/key words in combination with Boolean operators:

Biomarkers	Fat	Fat intake
Adipose tissue	Fatty acids	FFQ
Plasma	Lipids	Food diary
Plasma phospholipids		Recall
Plasma TAG		Duplicate diets
Erythrocytes PL		Weighed intake
Buccal cells		
Cholesteryl ester		

1.10.4.5 Results

Seventeen articles that matched with the inclusion criteria were identified. Some studies compared more than one biomarker. Five studies compared adipose tissue as biomarker with fat intake (Godley *et al*. 1996; Garland *et al*. 1998; Yli-Jama *et al*. 2001; Baylin *et al*. 2005; Cantwell *et al*. 2005). Erythrocytes were used in seven studies (Godley *et al*. 1996; Fuhrman *et al*. 2006; Sullivan *et al*. 2006; Sun *et al*. 2007; Wennberg *et al*. 2009;

Zhang *et al*. 2010; Jeppesen *et al*. 2012). The use of plasma phospholipids was examined in four studies (Ma *et al*. 1995; Anderson *et al*. 1996; Hodge *et al*. 2007; Thiebaut *et al*. 2009). Total plasma was reported in four studies (Baylin *et al*. 2005; Sullivan *et al*. 2006; Sun *et al*. 2007; Astorg *et al*. 2008). One study used plasma cholesteryl esters (Brunner *et al*. 2001).

The findings are summarised in **Table 1.5**. Most studies found no significant correlation between SFA and MUFA intake and those of the biomarkers. However, few exceptions reported weak correlations for SFA intake and an Italian study (Fuhrman *et al*. 2006) reported a surprisingly strong correlation between oleic acid intake and the proportion in erythrocytes. Most studies found positive correlations with PUFA, TFA, LA, EPA and DHA intakes. The findings were most consistent for EPA and DHA. Variable results were found for ALA, which implies that there may have been limitations in the dietary assessment procedures that may have overlooked dietary sources of ALA. Total plasma lipids appeared to yield comparable results to plasma PL and CE.

The OPTILIP study (Sanders *et al*. 2006a) which compared different ratios of long and short chain *n*-3 PUFA showed dietary EPA and DHA, but not ALA, to be reflected in changes in the proportions of EPA and DHA in erythrocyte lipids. With the exception of the MARINA and RISCK studies, which shall be reported on later in this thesis, no other large RCT were found that met the criteria for the randomized controlled trials.

Table 1.5 Correlation between dietary intake and biomarkers of fatty acid intake in previously reported studies.

Study	Tissue	No M/F	Correlation	SFA	MUFA	PUFA	TFA	16:0	18:0	OA	LA	ALA	EPA	DHA
Yli-Jama *et al*. 2001	Adipose	218 MF	Pearson				0.31**	-0.009	0.123	-0.111	0.49**	0.437*	0.609**	0.588*
Baylin *et al*. 2005	Adipose	196 MF	Pearson	0.04	0.06	0.51**	0.31**	0.1	0.05	0.15	0.52	0.51	-0.08	0.26
Cantwell *et al*. 2005	Adipose	84MF	Pearson				0.17				0.58*			
Garland *et al*. 1998	Adipose	140F	Spearman	0.16**	0.04	0.40**	0.40**	0.14	NA	0.12	0.37**	0.34**	NA	NA
Godley *et al*. 1996	Adipose	109 M	Spearman										0.38**	0.32**
Fuhrman *et al*. 2006	Erythrocytes	94PRE-F	Pearson	0.14	0.40**	0.17				0.45**	0.23*	0.14		
Fuhrman *et al*. 2006	Erythrocytes	104PM-F	Pearson	0.07	0.48**	0.39**				0.47**	0.39**	0.07		
Sullivan *et al*. 2006	Erythrocytes	53MF	Spearman										0.40**	0.39**
Wennberg *et al*. 2009	Erythrocytes	88M	Spearman					-0.02			-0.08	-0.3	0.42**	0.48**
Wennberg *et al*. 2009	Erythrocytes	92F	Spearman					0.04			0.09	0.12	0.46**	0.51**
Zhang *et al*. 2010	Erythrocytes	M44F8§	Spearman	-0.15	0.07	-0.08						0.19*	0.37**	0.16**
Godley *et al*. 1996	Erythrocytes	124M	Spearman										0.44**	0.41**
Jeppesen *et al*. 2012	Erythrocytes	2224MF	Pearson										0.4**	0.18**
Sun *et al*. 2007	Erythrocytes	306F	Spearman	0.12**	0.05	0.13**	0.43**	0.03	0.01	0.14**	0.24**	0.18**	0.38**	0.56**

Table 1.5 continued														
Study	**Tissue**	**No M/F**	**Correlation**	**SFA**	**MUFA**	**PUFA**	**TFA**	**16:0**	**18:0**	**OA**	**LA**	**ALA**	**EPA**	**DHA**
Baylin *et al*. 2005	Plasma	196 MF	Pearson	0.11	0.14*	0.39**	0.11	0.14*	0.01	0.32**	0.41**	0.39**	0.28**	0.31**
Sun *et al*. 2007	Plasma	306F	Spearman	0.16**	0.04	0.23**	0.3**	0.12**	0.06	0.12**	0.25**	0.23**	0.21**	0.48**
Sullivan *et al*. 2006	Plasma	53MF	Spearman										0.54**	0.48**
Astorg *et al*. 2008	Plasma	276M	Pearson								0.22**	0.06	0.24**	0.25**
Astorg *et al*. 2008	Plasma	257F	Pearson								0.19**	0.05	0.27**	0.27**
Hodge *et al*. 2007	Plasma PL	4439MF	Pearson	0.10**	0.26**	0.26**	-0.22	0.09		0.22**	0.20**	0.07	0.18**	0.40**
Ma *et al*. 1995	Plasma PL	1712M,1858F	Pearson	0.20**	0.07	0.44**		0.13**		0.13**	0.26**	0.26**	0.36**	0.55*
Anderson *et al*. 1996	Plasma PL	462M 117F	Pearson					-0.23**		-0.21	-0.04	-0.04	0.51**	0.49**
Thiebaut *et al*. 2009	Plasma PL	1114F	Spearman								0.11**	0.01	0.28**	0.36**
Brunner *et al*. 2001	Plasma CE	115M	Spearman	0.16		0.43**					0.38**		0.54**	
Brunner *et al*. 2001	Plasma CE	71F	Spearman	0.26*		0.50**					0.57**		0.23	

SFA=saturated fatty acids, MUFA=monounsaturated fatty, PUFA=polyunsaturated fatty acids, TFA=*trans* isomeric fatty acids, OA=oleic acid, LA=linoleic acid, ALA=alpha-linolenic acid, EPA=eicospentaenoic acid, DHA= docosahexaenoic acid, PL=phospholipid, CE=cholesteryl esters, PRE-F=premenopausal women, PM-F=postmenopausal women.

Statistical significance of correlation * $P<0.05$, ** $P<0.01$.

1.11 Digestion, absorption and metabolism of dietary fats

Most dietary fats are present as TAG, which is composed of a mixture of long chain and a small fraction of short- and medium chain fatty acids. The latter are hydrophilic and volatile and thus are directly absorbed by the intestinal capillaries and travel via the portal vein. On the contrary, emulsification is the central step for hydrolysis of long chain fatty acids, which are hydrophobic, to monoacylglycerol and free fatty acids (FFA) prior to absorption. This process is accomplished by three main stages: emulsification in the stomach, micelle formation facilitated by bile salt in the duodenum, lipolysis by pancreatic lipase in the duodenum and jejenum; uptake by the enterocytes in the ileum by passive transport (Bauer *et al*. 2005). The absorbed fatty acids and monoacylglycerols are re-synthesized into TAG in the enterocytes, secreted into lymph as chylomicrons and transferred via lymphatic vessels into the blood stream. To facilitate their transportation, they need to form lipoprotein complexes containing apolipoprotein B48.

Chylomicrons, which are large TAG rich lipoprotein particles, carry the esterified fatty acids from enterocytes to peripheral tissues such as muscle and adipose tissue, where TAG are hydrolysed by the action of lipoprotein lipase attached to the capillary endothelium. The resulting NEFA and glycerol, which travels through endothelial cells that make up blood capillaries, can either be used as an energy supply or stored as TAG. Chylomicron remnants are taken up by the liver by binding to receptors that recognise apolipoprotein B48.

In contrast to exogenous fat, which is transported by chylomicrons to tissues, endogenous fat arising from TAG synthesis in the liver is secreted as very low density lipoprotein (VLDL) which contains apolipoprotein B100 as opposed to apolipolipoprotein B48 in chylomicrons. VLDL reacts with lipoprotein lipase on endothelial cells where fatty acids are cleaved from the TAG. As TAG is removed from VLDL it decreases in size to form IDL, which may also contain apolipoprotein E, IDL particles without apolipoprotein E become substrates for hepatic lipase and are converted to LDL which contain apolipoprotein B100 as its sole protein. LDL is removed from circulation by the process of receptor-mediated endocytosis where apolipoprotein B100 binds to the LDL receptor and is taken up into cells. Most LDL receptors are expressed in the liver, which is thus the main site for removal of LDL. HDL is secreted from the liver and to some extent from the small intestine in a discoidal form low in lipid but as it acquires lipids in circulation it become spherical (Basso *et al*. 2003; Brunham *et al*. 2006). The main proteins in HDL are apolipoprotein A1 and A2. HDL particles acquire cholesterol and phospholipid from the liver and peripheral tissues. Mature HDL goes back to the liver although it can transfer cholesterol and phospholipid to TAG rich lipoprotein particles such as VLDL and IDL in exchange for TAG by the action of cholesterol ester transfer protein (Rader & Daugherty, 2008; Ratnayake & Galli, 2009).

The majority of fatty acids (~ 90%) circulating in the plasma are in the esterified form (TAG, PL, CE) and enclosed in lipoprotein particles. In the fed state, when insulin

levels are high NEFA released from chylomicron TAG by lipoprotein lipase (activated by insulin) are stored in adipose tissue or taken up by muscle for energy metabolism. In the fasting state, NEFA are released from adipose tissue by the action of hormone sensitive lipase (activated by adrenaline) and transported in blood bound to albumin where they can be taken up by tissues for oxidation to supply energy or can be resynthesized into TAG in the liver (Bergman *et al*. 1971; Coppack *et al*. 1992; Hellerstein, 1999).

The type of fat consumed influences the composition of these various lipid fractions. However, it is necessary to consider how each fraction changes in relation to dietary intake. TAG reflects intake over the past few hours and are subject to large variations (Durrington *et al*. 1977; Moore *et al*. 1977; Vessby *et al*. 1980). CE is marker of a longer-term intake to some extent (~3-5 days) (Zock *et al*. 1997, Zuijdgeest-van Leeuwen *et al*. 1999). Phospholipids are representative of intake over similar time periods to CE and are most frequently used lipid fraction in spite of low concentrations (Seppanen-Laakso *et al*. 2001; Zuijdgeest-van Leeuwen *et al*. 1999). Cell membrane PLs have been commonly used in nutritional studies as a marker of fat intake. It has been implied that fatty acids of cell membranes, which have a lifespan of 120 days, are a better indicator of fat intake than plasma fatty acids (Arab, 2003) particularly for DHA (Sun *et al*. 2007). Alternatively, adipose tissue is the marker of choice to mirror long-term fatty acids intake over years because of its slow turn over (Dayton *et al*. 1966; Hodson *et al*. 2008) with a half-life of approximately 6 months (Beynen *et al*. 1980;

Katan *et al*. 1997; Strawford *et al*. 2004). SFA and MUFA can be synthesized endogenously, while PUFA are almost solely exogenous and accordingly are superior biomarker of fat intake (Arab, 2003). There are other factors that influence efficiency of absorption and metabolism. These include, but are not limited to, TAG structure and type of fatty acid of interest. For instance, EPA and DHA from fish oils are incorporated at diverse rates into CE, erythrocytes and adipose tissue (Katan *et al*. 1997). The proportions of fatty acids in a lipid fraction as opposed to their absolute concentrations appear to be a more robust index of the quality of fat intake than traditional dietary assessment methods such as FFQ. Furthermore, they are increasingly being used to measure association between fatty acid intake and CVD risk in intervention and epidemiological studies (Harris *et al*. 2007; Khaw *et al*. 2012; Mozaffarian *et al*. 2013).

1.11.1 Factors influencing EFA metabolism

Metabolism of fatty acids including EFAs occurs in the mitochondria. Delta-5 (D5D) and delta-6 (D6D) desaturases and elongases are the key enzymes responsible for metabolism of LA and ALA to their respective metabolites (**Figure 1.3; P35**). A competition for these enzymes takes place between these two EFA with ALA being the preferred substrate over LA. Another fatty acid that is metabolised by the same desaturases is oleic acid and under normal physiological states, oleic acid metabolites are produced in negligible amounts only. As a consequence, high tissue and plasma levels of Mead acid (20:3*n*-9), which is a metabolite of oleic acid, is a well established

indicator of EFA deficiency that is used to distinguish the incidence of EFA deficiency in patients, experimental animals and in *vitro* studies (Siguel *et al*. 1987).

There are several factors known to influence desaturases and elongases activities. D5D and D6D are inhibited by SFA, cholesterol, TFA, alcohol, adrenaline and glucocorticoids. Aging, diabetes, oncogenic viruses and radiations, total fasting, protein deficiency and glucose rich diets reduces D6D activity. On the other hand, insulin, fat free diet and caloric restriction enhance D6D activity. Normal D6D activity requires pyridoxine, zinc and magnesium as co-factors. Disease states such as diabetes, hypertension, metabolic syndrome and hyperlipidaemia are all associated with decreased activities of D5D and D6D (Das, 2008).

1.12 Heritability

1.12.1 Heritability: definition and determination

Heritability is a simple measure of the significance of genetic factors in explaining the variation between human beings, permitting comparison of the same trait across populations and of different traits within a given population. Such comparisons can guide insights into the biology of the phenotype. In medicine and human genetics, estimates of heritability can be measured in both healthy and disease states to gauge the relative impact of genetic and environmental factors.

Heritability (h^2) is defined as the proportion of total variance in a population for a particular measurement, taken at a particular time that is attributable to differences in the magnitude of additive genetic values. Heritability is a ratio of variances: the denominator contains the total observed variation due to both genes and environment and the numerator contains variation that is due to additive genetic values in the population (Visscher *et al*. 2008).

Heritability relates mainly to the population under study, because both the variation in additive genetic factors and the environmental variance are population-specific. The key determinants of genetic variance are segregation in a population of the alleles that influence the trait, the allele frequencies, and the effect sizes of the variants and the mode of gene actions, which can differ across populations. Similarly, environmental variance can differ across populations. Therefore, the heritability in one population does

not necessarily predict the heritability of the same trait in another, although in practice it usually does (Visscher *et al*. 2008; Khoury *et al*. 1993).

Box 1 | Heritability and the partitioning of total variance

Observed phenotypes (P) of a trait of interest can be partitioned into a statistical model representing the contribution of the unobserved genotype (G) and unobserved environmental factors (E):

Phenotype (P) = Genotype (G) + Environment (E)

The variance of the observable phenotypes (σ^2_P) can be expressed as a sum of unobserved underlying variances (σ^2_G and σ^2_E):

$$\sigma^2_P = \sigma^2_G + \sigma^2_E$$

Heritability is defined as a ratio of variances, by expressing the proportion of the phenotypic variance that can be attributed to variance of genotypic values.

The genetic variance σ^2_G can be partitioned into the variance of additive genetic effects, of dominance genetic effects (interactions between alleles at the same locus) and of epistatic genetic effects (interactions between alleles at different loci). The additive genetic variance σ^2_a represents phenotypic variance which is transmissible from parents to offspring. This is the basis for most of the observable correlation among relatives and is used to compute heritability in the narrow sense h^2.

The environmental variance σ^2_E can be broken down into contributing factors including the variance that is common to specified groups σ^2_C, for example siblings, or variance unique to the individual σ^2_E.

$$\sigma^2_E = \sigma^2_c + \sigma^2_e$$

Total variance of the phenotype σ^2_P is the sum of the additive genetic and environmental variances

$$\sigma^2_P = \sigma^2_a + \sigma^2_c + \sigma^2_e$$

From this the heritability in the narrow sense, h^2 can be obtained, i.e. the ratio of the additive genetic variance (σ^2_a) to the phenotypic variance σ^2_P:

$$h^2 = \frac{\sigma^2_a}{\sigma^2_P}$$

Adapted from Vischer *et al*. 2008.

1.12.2 Determining heritability of metabolites

Statistical methodology to partition variation and to estimate heritability is well established (Neale *et al*. 2002). A high heritability implies that most of the variation that is observed in the population is caused by variation in genotypes. It indicates that, in the population, the phenotype of an individual is a good predictor of the genotype. A low heritability means that of all observed variation; a minute proportion is caused by variation in genotypes (Teucher, 2007; Lee, 2010).

As many metabolites are represented in overlapping pathways, correlation of metabolites is likely. To understand the correlation, principal components analysis (PCA) is a data reduction technique which can be used to decrease the large number of correlated variables into clusters of fewer uncorrelated factors using raw metabolite values. Linear structural equation modelling (SEM) is used to approximate the genetic and environmental components of variance in the scores for the principle components. Univariate twin analyses are performed, in which the phenotypic variance is decayed into additive genetic (A: additive effects of genes on multiple loci), common environmental (C: environmental effects shared by twins reared in the same family) and unique environmental effects (E: environmental effects unique to the individual). The ACE models assume that monozygotic (MZ) pairs share the same A genetic variance (completely correlated) and dizygotic (DZ) pairs share one-half of the A variance (have a correlation of 0.5). The C variance is believed to be the same for both MZ and DZ

twin pairs (the shared environment contributes equally to the correlations for MZ and DZ twins) (Teucher, 2007; Lee, 2010).

The significance of variance components A or C in the model is tested by dropping these parameters and comparing the fit of the models. The model fit is based on χ^2 tests, where a small χ^2 and a high *P*-value indicate a good fit. Parsimony is assessed by the Akaike information criterion, which corresponds to χ^2 with 2df. The model with the least Akaike information criterion is favoured. A likelihood ratio test is used to test whether a significantly poorer fit was obtained when removing parameters. Estimates of variance components are derived from the best fitting model and presented with 95% confidence intervals (CIs) (McCaffery *et al*. 2007).

1.12.3 Twin studies can distinguish genetic and environmental influences

The classical twin study is the definitive design for exploring the relative significance of genetic and environmental factors to traits and diseases in human populations. Twin studies utilize the unique degree of genetic and environmental sharing between the two types of twin pair; MZ twins (share a general set of genes) and DZ twins (share only 50% of their genes overall). Additionally, both types of twins share the same uterus, birth date, age and aspects of their early and later environment. These characteristics allow the population-level variation of traits and diseases to be broken up into genetic, shared environmental and random environmental components. Separating shared genes from the shared family environment as origins of family similarity is difficult to attain in any other practical family design. Application of quantitative analytical methods to

twin data aids in estimating the size of the contribution of these individual components of variation, to offer an estimate of heritability. Studies of twins have been used commonly to explore the heritability of common complex diseases. The outcomes have consistently revealed an important contribution from genetic variation to disease susceptibility. MZ twins are genetically alike. DZ twins are genetically indistinguishable from typical siblings, but the exact matching for age and date of birth is exclusive among family studies. For most complex diseases, age is vitally essential in disease expression. The shared family environment of twins offers a greater degree of matching for a range of environmental variables, both measured and unmeasured, which might contribute to the expression of diseases. Therefore, it is much easier to attribute phenotypic dissimilarities between twins to genetic rather than to environmental factors. A further advantage of studying twins is that the probability of non-paternity, an essential cause of misclassification in sibling studies, is diminished to approximately nil (Macgregor *et al*. 2000).

1.12.4 Application to biomarkers: genetic and environmental influences on plasma/adipose fatty acid proportions

It has been assumed in most studies that the biomarkers of fat intake are not influenced by genetic factors but this assumption may not be justified. As far as could be ascertained there is absence of reports on heritability of fatty acids measured as a biomarker. However, two studies were identified investigating the heritability of

branched chain fatty acids (Stewart *et al*. 1986) and heritability of metabolites (Shah *et al*. 2009).

Stewart *et al*. (1986) in 13 twin pairs examined the genetic influences on proportions of iso-branched fatty acids with even carbon number (iso-even) in sebaceous wax esters. They found very small intra-pair differences and a large inter-pair difference in the proportions of iso-branched fatty acids with even carbon number. This study was limited by a small number of subjects. Furthermore, the iso-branched fatty acids are derived from catabolism of valine, which is an essential amino acid. Thus, diet has made no contribution to the proportions of iso-branched fatty acids in sebaceous wax esters and their measurement is of no value with regard to biomarkers of fat intake. Whereas the study conducted by Shah *et al*. (2009) on heritability of metabolites was not performed on twin subjects and thus was omitted.

1.13 Candidate genes

A single nucleotide polymorphism (SNP) is a variation in base pair of DNA typically substitution of cytosine (C) to thymine (T) or adenine (A) to guanine (G) or a deletion within a gene. SNPs occur with high frequency (>1% population) and are not regarded as mutation and may or may not be functional i.e. carriage of the SNP does not affect the gene product. Two third of the SNPs are C-T or G-A (Ziegler & Konig, 2006). Fatty acid levels in the body, particularly PUFA, depend on both dietary intake and efficiency

of the metabolic pathways. The main enzymes involved in PUFA metabolism are D5D and D6D, as discussed earlier (*see* **Figure 1.3 P.35**). D5D and D6D are encoded by *FADS1* and *FADS2* genes respectively. SNPs at or near these genes are associated with increased or decreased levels of some of the LC-PUFA in the body. In MUFA metabolism, stearoyl-CoA desaturase is another enzyme that is influenced by SNPs in the encoding genes *SCD-1* and *SCD-5* (Paton & Ntambi, 2009). SNPs may either have functional effects or act as co-inherited markers of unknown functional sites in the gene.

Estimation of a high heritability of an observed phenotype indicates important genetic factors in its determination. The next logical step is to explore which genes might be responsible. Some gene variants have shown particularly strong signals in genome-wide studies of association with plasma fatty acid proportions. Several SNPs in the *FADS1-FADS2* gene cluster have been shown to be associated with plasma or erythrocyte phosphoglyceride LC-PUFA concentrations in genome-wide association (GWA) studies (Lemaitre *et al*. 2011; Tanaka *et al*. 2009). Associations of SNPs and haplotypes in this region have been shown to be associated with fatty acids in plasma (Martinelli *et al*. 2008; Schaeffer *et al*. 2006), erythrocyte membranes (Martinelli *et al*. 2008; Tanaka *et al*. 2009; Zietemann *et al*. 2010) and adipose tissue (Baylin *et al*. 2007). Such studies provided evidence that carriage of variants in the *FADS* gene cluster associates with plasma proportions of a number of LC-PUFA. *SCD-1* gene variants were found to be associated with distribution of body fat and insulin sensitivity (Warensjo *et al*. 2007).

There is a lack of association studies on *SCD-1* SNPs and proportions of fatty acids as biomarkers.

1.13.1 Fatty acid desaturases (FADS)

Levels of LC-PUFA are determined in the body by both dietary intake and metabolism of the precursor EFAs, LA and ALA, as a result of a successive desaturation and chain elongation (*see* **Figure 1.3; P.35**). D5D and D6D desaturases, which are membrane bound microsomal enzymes and consist of 444 amino acids, are the main enzymes involved in the conversion of *n*-3 and *n*-6 fatty acids to their LC-PUFA in the human body. The first step in the synthesis of *n*-6 fatty acids requires that D6D desaturates LA to GLA by the addition of an extra *cis* double bond at the position six of the chain. Then elongation of the existing fatty acid leads to the production of DGLA. Further desaturation by the action of D5D results in the formation of very LC-PUFA, AA, that is metabolized to produce eicosanoids or elongated and desaturated by the assistance of D6D enzyme resulting in the formation of 22:4*n*-6, 24:4*n*-6, 24:5*n*-6, and 22:5*n*-6. The production of LC-PUFA from *n*-3 fatty acids involves the desaturation of ALA by D6D to form stearidonic acid (18:4*n*-3), which is then elongated to form eicosatetraenoic acid (20:4*n*-3). After that a desaturation step by D5D takes place to give rise to EPA, which is either metabolized to produce eicosanoids or further elongated and desaturated to give rise to 22:5*n*-3, 24:5*n*-3, 24:6*n*-3 and 22:6*n*-3. The latter is a product of β-oxidation (Lattka *et al*. 2009).

Both D5D and D6D are crucial enzymes for the synthesis of LC-PUFA as well as cell membrane fluidity. The expression of desaturases enzymes occurs in all human tissues with higher rates in liver tissues followed by brain, heart and lung (Schaeffer *et al*. 2006). There are two genes coding for D5D and D6D in humans beings. These are fatty acid desaturase 1 (*FADS1*), which encodes D5D, and fatty acid desaturase 2 (*FADS2*), which encodes D6D. In 1999, first cloning of the human desaturases took place, while *FADS1* and *FADS2* genes were mapped in 2000 in the region 11q12-13.1 of chromosome 11 with head-to-head orientation (*see* **Figure 1.9**). This region was previously associated with complex diseases such as type 2 diabetes, osteoarthritis, asthma and allergy (Schaeffer *et al*. 2006). *FADS1* and *FADS2* genes share the same exon/intron organization, which are 12 exons and 11 introns. Around 500 SNPs identified in the *FADS1* and *FADS2* region and were recorded in the NCBI database (Lattka *et al*. 2009). Schaeffer *et al*. (2006) performed the first association study involving *FADS* polymorphism and fatty acids levels in serum PL and identified 11 SNPs with a highly significant association with *n-3* and *n-6* fatty acids except for docosapentaenoic acid (DPA, 22:5*n*-6) and DHA. The authors found that carriers of the minor alleles of (rs174544, rs174553, rs174556, rs174561, rs3834458, rs968567, rs99780, rs174570, rs2072114, rs174583, rs174589) express elevated levels of LA, eicosadienoic acid (20:2*n*-6), DGLA, and ALA and a low levels of GLA, AA, adrenic acid, EPA, and DPA (Schaeffer *et al*. 2006). A study investigating the association between levels of PUFA in serum PL with *FADS1, FADS2,* and *FADS3* SNP genotypes in Italian patients with CVD found that haplotypes showed a strong relationship with

AA levels in serum PL (P<0.0001) and LA, ALA and eicosadienoic acid in erythrocyte cell membranes (P<0.0001). Homozygotes and heterozygotes for the minor allele were associated with increased levels of LA, ALA, eicosadienoic acid and decreased levels of AA. The association of stearidonic acid (18:4*n*-3), EPA and DHA was not significant (Malerba *et al*. 2008).

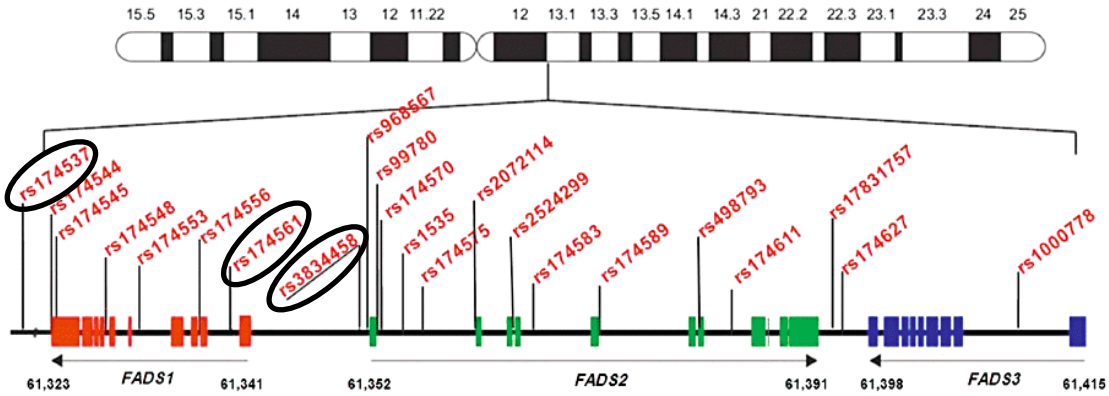
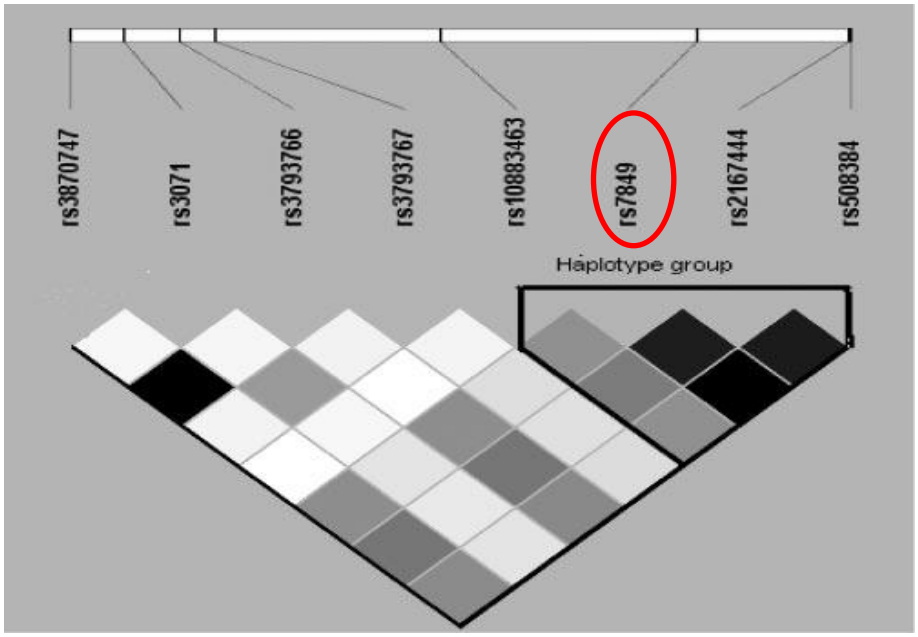


Figure 1.9 Location of *FADS1* and *FADS2* genes on chromosome 11.The SNPs investigated in this thesis are encircled. From (Glaser *et al.* 2011).

1.13.2 Stearoyl-CoA desaturase (SCD)

Stearoyl-CoA desaturase (SCD), a delta-9 desaturase, is the rate-limiting enzyme in the biosynthesis of MUFA from SFA aerobically in the endoplasmic reticulum (*see* **Figure 1.3; P.35**). Palmitoyl-CoA and stearoyl-CoA are the main substrates for the biosynthesis. There are two genes for the SCD that are expressed mainly in humans. *SCD-1*, which is expressed almost everywhere in the body and *SCD-5*, which is expressed mainly in the brain and pancreas. *SCD-1* is located on chromosome 10 (10q24.31) as shown in **Figure 1.10**, while *SCD-5* is located on chromosome 4 (4q21.22). *SCD-1* shares 85% homology with murine *SCD* genes. Studies on animals showed that mice deficient in *SCD-1* produce low body adiposity, improved sensitivity to insulin and were resistant to diet-induced obesity. Therefore, the block of *SCD-1* gene expression might be of beneficial effect in the treatment of metabolic diseases such as obesity and diabetes (Paton & Ntambi, 2009). Another study found that homozygotes of the rare alleles of rs10883463, rs7849, rs2167444, and rs508384 SNPs are associated with a lower BMI and waist circumference as well as enhanced insulin sensitivity (Warensjö *et al*. 2009). In contrast, research regarding the association between *SCD-1* SNP genotypes with fatty acid profile is lacking. However, a small number of investigations have been done on sheep (Garcia-Fernandez, 2009) and cattle (Milanesi *et al*. 2008; Kgwatalala *et al*. 2007). *SCD*-5 was not investigated in the present thesis because it is mainly expressed in brain and pancreas, unlike *SCD-1*, which is widely expressed in the body.



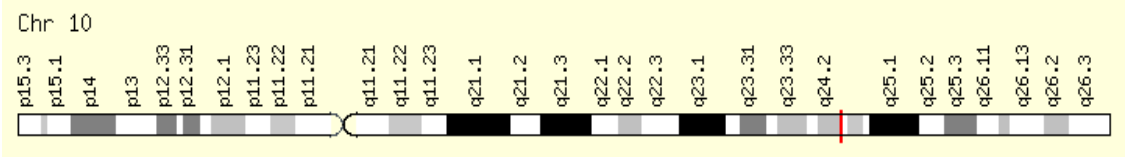


Figure 1.10 Location of *SCD-1* gene on chromosome 10. The locations of the SNP investigated in this thesis are indicated by the red circle and line. From (Warensjö *et al*. 2009; http://www.genecards.org/cgi-bin/carddisp.pl?gene=SCD).

1.14 Aims and Objectives

The main aim of the present thesis was to test the reliability of the use of biomarkers in reflecting fat intake where diet had been modified in large randomized controlled trials. The second aim was to assess the effect of genetic factors on these biomarkers and specifically any effects on genes encoding fatty acid desaturase enzymes, *FADS1, FADS2* and *SCD-1*.

It was hypothesised that biomarkers would act as reliable markers of purely exogenous fatty acids i.e. PUFA and TFA and a poor marker of endogenously synthesised fatty acids i.e. SFA and MUFA

It was, also, hypothesised that genetic variation in *FADS1, FADS2* and *SCD-1* would modulate proportions of these fatty acids as biomarkers of intake.

The answers to the following questions were explored in this thesis:

To what extent does adipose tissue, plasma and erythrocyte phospholipid fatty acid composition change in response to varying the intake of specific fatty acids?

How much of the variance in fatty acid composition of adipose tissue and plasma total lipids can be explained by additive genetic effects?

Do variations in known SNPs for *FADS1*, *FADS2* and *SCD-1* influence the proportion of unsaturated fatty acids in adipose, total plasma and erythrocyte lipids?

Chapter 2: Materials and methods

This chapter provides a description of the methods used, the principal underlying them and a justification for the choice of analytical methods applied and the statistical approaches employed.

2.1 Lipid analysis

2.1.1 Determination of adipose tissue fatty acid composition

Several methods were initially evaluated these included a) extraction of the total lipids with chloroform:methanol, removal of the solvent and trans-esterification with sodium methoxide; b) extraction of the adipose tissue in hexane and removal of the water with anhydrous sodium sulphate and trans-esterification with sodium methoxide; c) acid catalysed interesterification as described below.

Reference:

Direct transesterification of all classes of lipids in a one-step reaction (adapted from Lepage, G. and C. C. Roy (1986)

Principle: The lipids are transesterified with methanol using HCL as catalyst. Toluene is added to the reaction mixture to facilitate the extraction of non-polar lipids. The reagent is a mixture of methanol in toluene (80:20 by volume), which solubilises the lipids, and contains HCL which catalyses the methylation of fatty acids to form methyl esters. After refluxing the mixture, potassium carbonate solution is added to facilitate the separation of a toluene rich supernatant, which contains the methyl esters. The latter

is collected for gas chromatography on a polar capillary column that separates the volatile fatty acid methyl ester (FME) based on molecular weight and unsaturation.

Materials for fatty acids extraction from adipose tissue

Item	Supplier
Toluene Analytical reagent grade (AR)	BDH, Poole, Dorset
Hexane Puriss	BDH, Poole, Dorset
0.5 N HCL in methanol	Superlco obtain through Sigma-Aldrich
6% potassium carbonate in distilled water	BDH, Poole, Dorset
Fatty acid methyl standards 189-1, 189-2,189.3	Sigma-Aldrich, BDH, Poole, Dorset
MaxEPA	Seven Seas Ltd, Hull, UK
Column BPX70 SGE (60m x 250μm x 0.25μm)	SGE Analytic Science, Milton Keynes
Gas chromatography	Agilent 7890A GC; Agilent Technologies

Methylating reagent HCL methanol:toluene (4:1 by volume) prepared by adding 20ml toluene to 80ml 0.5 N HCL in methanol solution (Sigma).

Standards preparation

External standards used were fatty acid methyl standards of Sigma 189-1, 189-2 and189-3 dissolved in hexane 1mg/ml and methyl esters of MaxEPA as an external standard for EPA and DHA dissolved in hexane 1mg/ml.

Sample preparation

Adipose tissue (~5mg) was transferred into a clean culture tube fitted with Teflon lined screw-cap (16x25mm). 2 ml of methylating reagent was added and tube sealed and incubated in water bath for 2hrs at 60°C. Sample was left to cool down and 5ml of 6% potassium carbonate was added. Sample was vortex mixed and centrifuged at 2500rpm for 10min. The upper layer was then collected into an amber GLC vial and 0.5ml toluene was added. Samples were then analysed by GLC and each sequence contained a sample blank, a set of standards and a quality control sample in addition to the samples.

Fatty acid methyl esters (FAME) were separated on an Agilent 7890 Gas Chromatograph (Agilent Technologies) with electronic pressure control fitted with a flame ionisation detector (FID) with a 60m BPX70 column (SGE). Hydrogen was used as the carrier gas at a constant flow rate of 1.37ml/min. Peaks for the FAME were identified by comparison of the retention times with standards of known composition obtained from Sigma Aldrich (Individual fatty acids were identified by reference to standards obtained from Sigma (FAME standard 189-1,189-2, 189-3) and a secondary reference standard of MaxEPA (Seven Seas Ltd, Hull, UK) for LC *n*-3 PUFA.

GC was Agilent 7890A GC; Agilent Technologies operated under the following conditions:

Injector	
Type	Front injector
Injection volume	3μL
Inlet detector	
Injection mode	Split 50:1
Inlet temperature	250ºC
Gas	
Carrier gas	Hydrogen at 1.37ml/minute
Make up gas	Nitrogen
Column	
Column	BPX70 SGE (60m x 250μm x 0.25μm).
Oven	
Temperature/programme	Initial temperature 180ºC, held for 50 minutes.
Run time	50 min
Detector	
Type	Flame ionization detector
Detector temperature	280ºC
Data acquisition rate:	20Hz

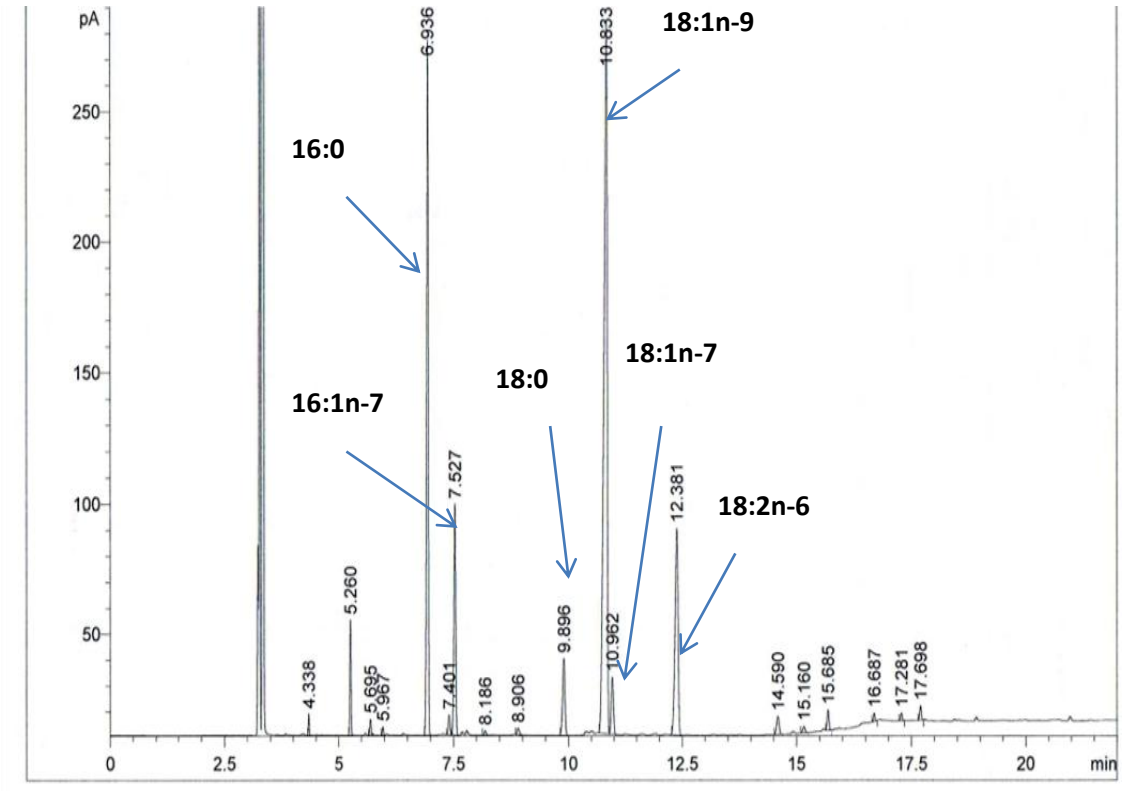


Figure 2.1 Fatty acid chromatogram of adipose tissue lipids. Initial peak at a retention time of ~3 min is solvent peak followed by individual fatty acids methyl esters. The run was isothermal on a 60 m BPX70 column with hydrogen carrier gas flow rate of 1.37 ml/m

2.1.2 Determination of plasma fatty acid composition

Reference:

Method adapted from Lepage, G. and C. C. Roy (1986).

Principle: Total lipids are transesterified with HCL as a catalyst in methanol:toluene solution and the FAME analysed by gas chromatography in the presence of a 15:0 as an internal standard. The reagent is prepared by added acetyl chloride to methanol.

Materials for fatty acids extraction from plasma

Item	Supplier
Toluene AR	BDH, Poole, Dorset
Hexane Puriss	BDH, Poole, Dorset
Methanol AR	BDH, Poole, Dorset
Acetyl chloride AR	BDH, Poole, Dorset
6% potassium carbonate in distilled water	BDH, Poole, Dorset
Internal standard: pentadecanoic acid	Sigma-Aldrich, BDH, Poole, Dorset
External standards: Sigma 189-1, 189-2,189.3	Sigma, BDH, Poole, Dorset
MaxEPA	Seven Seas Ltd, Hull, UK
BPX70 column (25 m X 220 μm X 0.25 μm)	SGE Analytic Science, Milton Keynes
Gas chromatography	Agilent 7890A GC; Agilent Technologies

Internal standard 50μg pentadecanoic acid/ml methanol:toluene (4:1 by volume)

Standard preparation

Preparation of the internal standard took place by adding 5ml of 1mg/ml internal standard in methanol to 75ml methanol. Toluene was then added to make-up to 100ml. The mixture was then chilled on ice in a conical flask and acetyl chloride (10ml) added drop-wise while swirling the flask in a fume cupboard. The reagent was allowed to cool before use and was stable for up to two weeks at room temperature.

Authentic reference standards were used. FAME standards of Sigma 189-1, 189-2 and 189-3 dissolved in hexane 1mg/ml and FAME of MaxEPA as a reference standard for DHA and EPA dissolved in hexane 1mg/ml.

Sample preparation

Sample preparation took place by pipetting 0.1ml plasma into a tube fitted with Teflon lined screw cap (16x25mm) using a positive displacement pipette. 2.2ml of internal standard mixture were added into the tube, which is then sealed and heated in water bath for 2hrs at 60°C. After cooling down, 5ml of 6% potassium (or sodium) carbonate were added and sample centrifuged at 2500rpm for 10min. The upper phase was then transferred into an amber vial for GLC analysis.

FAMEs were separated on an Agilent 7890 Gas Chromatograph (Agilent Technologies) fitted with a FID with a 25m BPX70 column (SGE). Peaks for the FAMEs were identified by retention time with standards of known composition obtained from Sigma Aldrich (Individual fatty acids were identified by reference to standards obtained from

Sigma, FAME standard 189-1,189-2, 189-3) and a secondary reference standard of MaxEPA (Seven Seas Ltd, Hull, UK) for LC *n*-3 PUFA.

Calculations

Fatty acid composition = area of fatty acid of interest ÷ (total area –area of internal standard)

GC was Agilent 7890A GC; Agilent Technologies operated under the following conditions:

Injector	
Type	Front injector
Injection volume	5μL
Inlet detector	
Injection mode	Split 50:1
Inlet temperature	240ºC
Gas	
Carrier gas	Hydrogen at 1 ml/minute
Make up gas	Nitrogen
Column	
Column	BPX70 SGE (25 m x 220 μm x 0.25 μm).
Oven	
Temperature/programme	Initial temperature 160ºC, held for 4 min, then ramped 10ºC/min for 10 min to 200ºC, then 40ºC/min for 10 min to 240ºC.
Run time	29 min
Detector	
Type	Front Ionisation Detector (FID)
Detector temperature	250ºC
Data acquisition rate:	20Hz

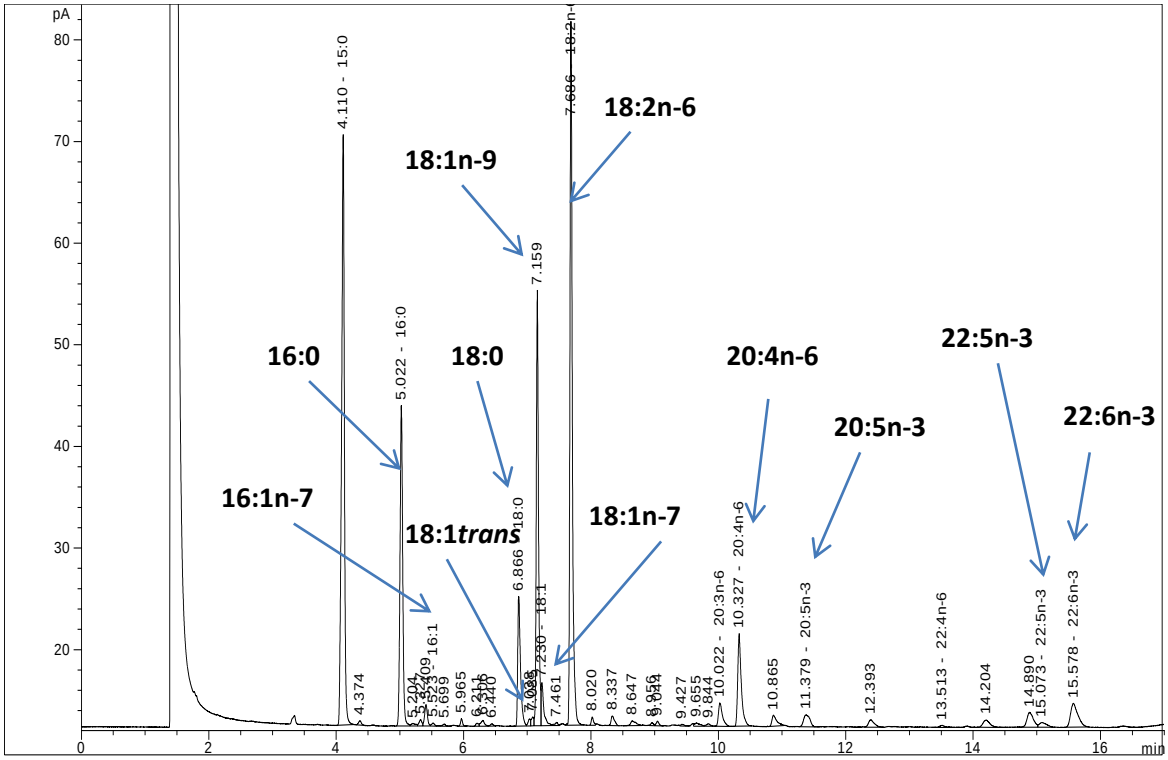


Figure 2.2 Fatty acid chromatogram of plasma lipids. The peak at retention time ~1.5min respresent the solvent peak followed by FAME. Analysis conducted on 25m BPX70 column using a temperature programme.

2.1.3 Determination of erythrocyte phospholipids

Reference:

Rose & Ocklander (1965) as modified by Sanders *et al*. 1997.

Determination of erythrocyte phospholipids was done by Mr Robert Gray (Diabetes and Nutritional Sciences Division, King's College London).

Principle: Fatty acids present as acyl moieties in membrane bound lipids are converted (transesterified) to FAME and analyzed by capillary column FID gas chromatography. Erythrocytes were first washed with isotonic saline to remove plasma and then the lipids extracted with a mixture of isopropanol:chloroform (11:7 by volume). The extracted lipid sample was stored at -20°C until analysis when FAMEs were prepared by transesterification with sodium methoxide which reacts with phosphoglycerides but not sphingomyelin.

Materials for fatty acids extraction from erythrocyte phospholipids

Item	Supplier
Toluene AR	BDH, Poole, Dorset
Hexane Puriss	BDH, Poole, Dorset
Methanol AR	BDH, Poole, Dorset
Acetyl chloride AR	BDH, Poole, Dorset
6% potassium carbonate in distilled water	BDH, Poole, Dorset
Internal standard: pentadecanoic acid	Sigma-Aldrich, BDH, Poole, Dorset
External standards: Sigma 189-1, 189-2,189.3	Sigma, BDH, Poole, Dorset
MaxEPA	Seven Seas Ltd, Hull, UK
BPX70 column (25 m X 220 μm X 0.25 μm)	SGE Analytic Science, Milton Keynes
Gas chromatography	Agilent 7890A GC; Agilent Technologies

Cold saline (0.89% NaCl; 8.9g NaCl/L containing 40mg EDTA/L)

Sample preparation

Blood was collected into EDTA coated tubes (BD vacutainer 367873). Plasma was removed and the erythrocytes were washed three times with ice-cold isotonic saline (8.9 NaCl/L water containing 40mg EDTA/L). 1ml of packed cells was then haemolysed by the addition of 1ml distilled water and ice-cold isopropanol (11 volumes). After standing for 30min on ice, 7ml chloroform was added followed by vortex mixing. The sample was allowed to stand for a further 30min before centrifuging. The lipid extract was carefully transferred into a clean glass culture tube fitted with a Teflon liner and

stored at -20°C until fatty acid methyl esters were prepared for analysis**.** Prior to analysis solvents were removed in a centrifugal evaporator and the lipid extract trans-methylated with sodium methoxide in methanol. FAME were analysed by capillary GLC on a 25m BP70X capillary column (SGE, Milton Keynes).

GC was Agilent 7890A GC; Agilent Technologies operated under the following conditions:

Injector	
Type	Front injector
Injection volume	5 μL
Inlet detector	
Injection mode	Split 50:1
Inlet temperature	240ºC
Gas	
Carrier gas	Hydrogen at 1ml/minute
Make up gas	Nitrogen
Column	
Column	BPX70 SGE (25 m x 220 μm x 0.25 μm).
Oven	
Temperature/programme	Initial temperature 160ºC, held for 4 min, then ramped 10ºC/min for 10 min to 200ºC, then 40ºC/min for 10 min to 240ºC.
Run time	29 min
Detector	
Type	FID
Detector temperature	250ºC
Data acquisition rate:	20Hz

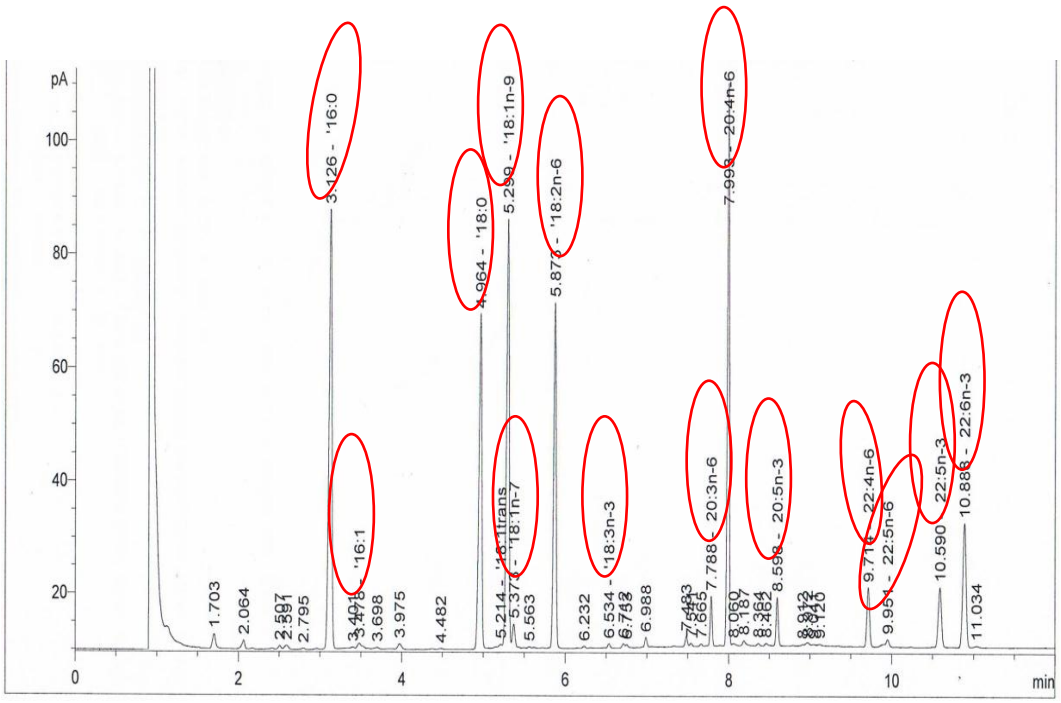


Figure 2.3 Fatty acid chromatogram of erythrocyte phosphoglycerides. The peak at retention time ~1min respresent the solvent peak followed by FAME. Analysis conducted on 25m BPX70 column using a temperature programme.

2.2 DNA extraction and SNP genotyping

DNA extraction from MARINA samples was performed by Dr Aseel AlSaleh. DNA extraction from TwinsUK samples was performed by staff at KCL Department of Twin Research (DTR). Buffy coats removed from blood samples were stored in EDTA at -20°C. Genomic DNA was extracted from 200µl using an Illustra blood genomic prep mini spin kit (GE Healthcare, Amersham, UK) according to manufacturer's instructions. Genotyping was performed on the 310 participants for whom DNA was available by KBiosciences (Hoddesdon, UK), using the KASPar system. Genotype accuracy, as assessed by inclusion of duplicates in the array was 98% and negative controls (water blanks) were included on each plate. The mean genotyping success rate was 97.7% (95.8-99.7%).

2.2.1 DNA extraction: Materials

DNA was extracted from the buffy coats using the Illustra blood genomic prep spin kit, (GE Heathcare, UK).

2.2.1.1 Materials for DNA extraction

Item	Supplier
Proteinase K	GE Healthcare, UK
Lysis buffer	GE Healthcare, UK
Wash buffer	GE Healthcare, UK
Elusion buffer	GE Healthcare, UK
Illustra blood mini column	GE Healthcare, UK
Collection tubes	GE Healthcare, UK
96-well microplates	Qiagen, Crawley, Surrey, UK
Sterile plate covers	Qiagen, Crawley, Surrey, UK

2.2.1.2 Equipment

Item	Supplier
Microcentrifuge that accommodates 1.5 ml microcentrifuge tubes	DJB Labcare Ltd, Buckinghamshire, UK
Water bath or heat-block for 70^{o}C incubation	Grant Operation
Vortex mixer	Fisherbrand, Fisher Scientific, UK

2.2.2 DNA extraction procedure

DNA extraction from buffy coats of 310 MARINA subjects was carried out using an Illustra blood genomic prep mini spin kit (GE healthcare, UK). Buffy coats in EDTA had been frozen since preparation of blood samples. Genomic DNA was purified from 200µl buffy coat. This process was carried out according to manufacturer's instructions. This includes the below steps:

1- **Blood cell lysis**: 20µl of Proteinase K was added into the bottom of a 1.5ml microcentrifuge tube. This digests protein, removing contamination and inactivating nucleases that might otherwise degrade the DNA during purification. This was followed by adding 200µl of the buffy coat and then 400µl lysis buffer into the same tube. The tube was mixed well for 15sec and incubated at room temperature for 10min.

2- **Genomic DNA binding:** a mini column was assembled in a collection tube. The sample was loaded on to the centre of the column and was centrifuged. The flow through in the collection tube was discarded. The column was placed back inside the collection tube.

3- **Wash:** to ensure complete cell lysis and to denature any residual protein; 500µl of lysis buffer was added to the column and centrifuged. And again, flow through in the collection tube was discarded.

4- **Wash and dry:** into the column, 500µl of wash buffer was added. This was centrifuged for 3min. The collection tube and flow through were discarded.

5- **Elution:** the purification column was transferred into a fresh DNAse-free microcentrifuge tube. Followed by adding 200µl of 70°C preheated elusion buffer on the centre of the column. This was centrifuged for 1min. Purified genomic DNA was stored at -20°C.

2.2.3 Quantitation of DNA samples

The quality of the DNA samples was checked by NanoDrop (DNA quality analyses). NanoDrop accurately measures dsDNA samples up to 3700ng/µl without dilution. It automatically detects the high concentration and utilizes the 0.2mm path length to calculate the absorbance. For NanoDrop spectrophotometry, 1.0µl of sample was used to quantify DNA concentration. A blank of nuclease-free water, was used to optimise the instrument. Sample pedestals were first cleaned with nuclease-free water and ethanol prior to use.

2.3 Blood Analytic methods

2.3.1 Fasting lipids for RISCK, CRESSIDA and MARINA studies

Analyses of total cholesterol (TC), HDL-C and triacylglycerol (TAG) were carried out at KingsPath, King's College Hospital. Precision estimates were based on each sample assayed 2 times per run, 2 runs per day, for at least 10 days and computed according to CLSI document EP05-A2, Evaluation of Precision Performance of Quantitative Measurement Methods: Approved Guideline. Reagents were supplied by Bayer Diagnostics Europe Ltd. (Newbery, Berks, UK). TC was determined using an enzymatic method using cholesterol esterase, cholesterol oxidase and peroxidase in a chemiluminescent reaction to produce a red quinoneimine dye. The increase in absorbance was measured as an endpoint reaction at 505/694 nm. Inter-assay coefficients of variation (CV) were 1.1, 1.5 and 1.0 at 3.9, 5.2 and 5.7 mmol/L respectively. HDL-C was analysed using a two-step automated procedure (Bayer Advia Direct method, Bayer Diagnostics Europe Ltd, Newbery, United Kingdom). CVs were 2.2, 2.1 and 2.5 at 0.91, 1.39 and 1.95mmol/L respectively. TAGs were measured using an enzymatic assay (The Bayer Advia method, Bayer Diagnostics Europe Ltd, Newbery, United Kingdom). CVs were 2.5 and 1.5 at 1.32 and 2.36 mmol/L respectively. LDL cholesterol was calculated using the Friedwald formula if fasting plasma TAG concentrations are < 4.49 mmol/L. Formula used:

LDL-C = Total Cholesterol - HDL-C - (TAG / 2.2)

2.4 Carotid intimal mediated thickness (CIMT)

CIMT was measured by trained ultrasonographer in the participants at the final visit. CIMT is a non-invasive method for measuring carotid atherosclerosis. These measurements were not part of the contract but were funded by the National Institute of Health Comprehensive Biomedical Centre at Guy's and St Thomas' Hospital under the Atherosclerosis theme. Images of the left and right common carotid arteries were obtained by high resolution ultrasound at the endpoint visit. CIMT is measured from digitized images obtained in diastole of the near and far walls of both common carotid arteries 1-2cm proximal to the flow divider. This method is recognized as providing a robust measure of IMT with reproducibility higher than that in other segments.

The two dimensional scanning measures both left and right carotid systems, including the common carotid artery (CCA), carotid bifurcation and internal and external carotid artery (ICA and ECA). The carotid system was scanned longitudinally and cross-sectionally. Plaque was defined as a thickness over 1.5 mm, and classified soft and hard plaque, according to echo characteristics. Soft plaque is hypo-echogenic, where calcification is less than 50%. The hard plaque is calcified plaque with hyper-echoicones over 50%.

The measurement of CIMT was performed in the CCA, 1-2cm proximal from the bifurcation. The IMT was determined as the distance from the leading edge of the first echogenic line to the second echogenic line, which present the lumen-intima interface and the medial-adventitia interface respectively. The IMT was measured on both anterior and posterior walls, using callipers, and determined from the average of the two

readings. Doppler colour and spectral analysis was performed to assess the haemodynamic state. For documentation, the static image of all plaque was taken. In order to minimise variability during the cardiac cycle, the longitudinal image of CCA was frozen at the top of R wave of the ECG and stored for measuring diameter and IMT of the CCA. The edge detecting software Carotid Analyzer (Medical imaging Application LLC, USA) was used to measure the diameter of CCA and CIMT.

2.5 Statistical methods

All statistical analysis was performed using SPSS versions 17.0 and 20.0 for Windows (SPSS Inc, Chicago, IL, USA) unless otherwise specified.

2.5.1 Linear regression modelling

Linear regression is a statistical method aimed to test and to describe the linear relationship between two or more variables. The regression coefficient describes the angle of the regression line and reflects the amount of variance of the dependent variable that is explained by the variation of the independent variable (Boomsma *et al*. 2002). Linear regression modelling was used to compute EPA and DHA intake of the twin subjects using plasma proportions of these fatty acids and intakes data from the MARINA study by applying the equation of the regression line.

2.5.2 Adjustment for covariates

In a multivariate analysis, a variable or variables with known effect that is used to examine the impact of the variables of interest independent of those known effects. In the present thesis the inclusion of age, gender, BMI and ethnicity was taken into account when performing the main analysis to test the effect of the independent variable. Multivariate analysis can be defined as the inclusion of two or more dependent variables simultaneously (Boomsma *et al*. 2002).

2.5.3 Analysis of variance (ANOVA)

ANOVA is a statistical technique applied to test the null hypothesis that the mean values of two or more groups are equivalent. The variance around the means in groups is compared with the variance of the group means. In genetic applications, the variance between families is compared with the variance within families (Boomsma *et al*. 2002).

2.5.4 Generalized estimating equations (GEE)

For related individuals, conventional statistical analyses lead to inflated significance. Dependency of the observations within twin pairs was accounted for by the use of the Generalized Estimating Equations (GEE) procedure (Trégouët *et al*. 1997) in which both MZ and DZ twins can be used in tests of association. The approach accounts for dependency of the observations within pairs and yields unbiased standard errors and *P*-values. Association analyses in TwinsUK subjects were performed by members of the KCL Twin Research Group at the DTR.

2.5.5 Principal component analysis (PCA)

PCA was used to reduce the large number of correlated variables into clusters of fewer uncorrelated factors using raw metabolite values without removal of outliers. The factor with the maximum 'eigenvalue' accounts for the largest amount of the variability within the data set. Standardized residuals determined for each metabolite from linear regression models adjusted for age, sex and BMI are used as inputs for PCA. PCA using residuals is recommended when the units for each variable vary considerably in size. Factors with an eigenvalue were identified based on the frequently used Kaiser criterion. Varimax rotation is then applied to produce understandable factors. Metabolites with a factor load are reported as composing a given factor, as is commonly used as a random threshold. Scoring coefficients are then used to calculate factor scores for each individual (consisting of a weighted sum of the values of the standardized metabolites within that factor, weighted on the factor loading calculated for each individual metabolite). These factor scores are then used to calculate heritabilities for each factor with suitable software (Shah *et al*. 2009).

2.5.6 Structural equation modelling using Mx software

Linear structural equation modelling also known as covariate modelling, is a technique that estimates regression coefficients (parameters) between latent (unobserved) and observed variables. These estimates diminish the dissimilarity between the covariance structure of the observed data and that predicted by the model. It was used to estimate the genetic and environmental components of variance which decays variations in a

given phenotype into additive genetic effects (A), environmental effects shared in common (C) and non-shared environmental effects (E) using the Mx software package (Neale *et al*. 2002). The goodness-of-fit analysis was completed by calculating the difference between the model fitted and the fully saturated model. Adjustment allowing for differences in age and BMI were done using the linear regression analysis module within SPSS and save the residuals as a new variable. The distribution of the residual was then checked for normality and where appropriate was log-normalised before further analysis (Mohammed *et al*. 2005; Teucher *et al*. 2007; Boomsma *et al*. 2002).

Box 2.1| Step by step data analysis using Mx software

- Data:

1. Data need to be restructured (i.e. the 2 twins treated as one individual)

Note: restructuring the data using the SPSS software:

Data »» restructure »» a data wizard will come up

- Chose: restructure selected cases into variables (middle option) and click next
- Identified variable: Twin ID, index variable: order
- Cases to variables: sorting data
    - Yes: if data not sorted
    - No: if data sorted, i.e. 1st born followed by 2nd born as in the TWIN data file, so we selected this option
- Click next
- Cases to variables: options
    - Select "group by index" and click next and then finish

2. Check normality of the data
    1. Use scatter plot to check normality of the data
    2. Correlation between FA and age for example
    3. Histogram
    4. Skewness
3. Transform the data if required
    1. Log transformation
    2. Reciprocal is another transformation (-1/FA)

Note: always log/transform the data then regress

4. Regress possible covariates
    1. Age
    2. Gender
    3. BMI
    4. Total cholesterol, for example TC was regressed for the plasma fatty acids

Note: regression of the covariates using the SPSS software:

Analyze »» regression »» linear regression OR
Analyze »» general linear model »» univariate
In both cases new variable will be created in a new column

Preparing the data file for analysis using Mx software

1. Replace missing data with (.) or large number (999)
2. Twin ID **»» family id**
3. Check (min/max, SD)
    - If SD<1, multiply the variable by 10, because Mx can't deal with values <1
        i. 0.9 is ok, but if smaller than 0.5
    - If min is close to zero, add 1 to the variable to avoid –ve values
- Then restructure the data file as detailed in step 1
- Save file as a fixed format (comma separated file (.CSF) or fixed ASCII (.dat)

- Script
    - saturated model
        - reveals correlation of MZ and DZ without making any prediction of genetics or environmental influences
        - REC file = name of the file as saved (e.g. trial.dat or oleic.dat)
        - Labels = order of the variables in the file (famid age FA1 FA2 zyg)
        - data input = number of variables (e.g.5)
        - missing = the label that was given to the missing values (999)
        - O= MZ correlation P= DZ correlation
        - unace= univariate ACE
    - looking to non-sig difference between predicted and reality
    - using excel, calculate the *P*-value
        - using the CHIDIST function, insert Chi sq and df to compute *P*- value
    - we can't have a model dropping E. E is always significant
    - if the CI overlapping or contains zero, then it is not significant

- Some interpretation of the results
    - E is very important and always significant
    - when values of A and C are too close then
        - It is familial effect (A+C) and there is not enough power to detect genetic, but together familial influence is bigger than random variation
        - Significant MZ correlation implies genetic influence
        - If MZ corr & DZ corr is not significan, then we can't expect genetic effect
        - E tests if we can drop C & A together
        - Genetics can only be detected if and only if MZ corr above 0.5 & DZ corr is half of that

2.5.7 Haplotype analysis using THESIAS software

Statistical reconstruction of haplotypes in unrelated individuals, for whom phase is unknown, is possible when SNPs are in strong linkage disequilibrium (LD). Inter-locus linkage disequilibrium based on observed numbers of diplotypes was established using CubeX software (Gaunt *et al*. 2007), available online at:

(http://www.oege.org/software/cubex/) and represented in a correlation matrix as Lewontin's $\boldsymbol{D'}$ and squared correlation $\boldsymbol{r}^2$ measures between each pair of SNP loci. Strong LD between the SNPs is indicated by r^2 >0.8.

Reconstruction of haplotypes of the three SNPs at the *FADS1 FADS2* locus (rs174537-rs174561- rs3834458) was based on MARINA participants for whom all genotypes were available, to avoid errors resulting from missing data. Analysis of haplotypes was performed using the graphical JAVA interface of the THESIAS software package (Trégouët & Garelle, 2007), (available online at: http://ecgene.net/genecanvas/). This program is based on the maximum likelihood model linked to the stochastic expectation and maximization (SEM) algorithm (Trégouët *et al*. 2004) and is used to statistically reconstruct haplotypes in unrelated individuals and perform haplotype-based association analysis of phenotypes. Covariate-adjusted haplotype effects can be investigated.

LD and haplotype analysis was performed by Dr Sandra O'Dell.

Box 2.2| THESIAS haplotype analysis procedure

- Load the .txt data file. Click on Menu/Open file.

Results 1: Haplotype frequencies (null option)

- Select the polymorphisms to be studied.

The genotypes of the three SNPs are in 3 columns L to R corresponding to the txt. file. Genotypes are designated 11, 12 and 22. This first step is necessary to determine the haplotypic structure derived from the studied polymorphisms independently of any other phenotype/covariates.

- Press the Run button with the **Null** option.

In the Output Window information is displayed on:
- Number of individuals with and without missing data
- Allele frequencies of the polymorphisms with the corresponding test for Hardy-Weinberg equilibrium
- Haplotype frequency estimates under the assumption of no Linkage Disequilibrium (LD).
- Haplotype frequencies estimates under the assumption of LD

Results 2: Log likelihood no haplotype

- Select the **Quantitative** option

The main screen changes to show an additional panel in the right part of the screen which provides a list of the inferred haplotypes sorted by decreasing frequencies (Fq).

- Note only the top reference haplotype is checked in the RH panel.
- Once the additional right panel is activated, select the location of the **quantitative Phenotype** variable and add the variables as **Covariates**.

The plasma FA phenotypes (baseline) are in numbered columns L to R corresponding to the txt. file.

The covariates ethnicity, gender, age and BMI are in numbered columns L to R corresponding to the txt. file.

- Press Run.

In the Output Window are displayed:
-The observed phenotypic mean with its standard error.
-The intercept of the linear model. In absence of covariate and haplotype effects, it corresponds to half of the observed phenotypic mean.
-The estimated regression parameter associated with the covariate is then given.

-The log-likelihood of the data (without haplotype effect but here with 4 covariates) is given with its corresponding degree of freedom (df).

Results 3: Haplotype effects

- Check Estimation of haplotype effects
- Check the haplotype (s) in the haplotype list in the RH panel with frequency >5%.
- Press Run.

The Output Window now gives the estimated regression parameters characterizing the association between haplotype and the phenotype, compared to the most frequent haplotype. In the case of a quantitative phenotype, a linear formulation is used to link the haplotype to the phenotype under the assumption of additive effects. The order in which the haplotypes are displayed is based on the order polymorphisms are read in the datafile. Each haplotype is given a number that corresponds to its rank in the list of haplotype sorted by decreasing frequency. Haplotype 1 (the most frequent haplotype) always corresponds to the intercept of the regression model. Haplotype 2 is the second most frequent haplotype etc. The residual standard error is then given.

One minus the square of the ratio of the residual standard error over the global standard error provides an estimate of the **percentage of phenotypic variance explained by the covariates and the haplotype effects.**

The regression parameter associated with the covariate is then listed and followed by the **log-likelihood of the data with df.**

The **log-likelihood of the data (without haplotype effect but with covariates) with corresponding df** was produced in Results 2.

The Likelihood Ratio Test (LRT) for haplotype-phenotype association is twice the difference between the log - likelihood under the null (Results 2) and the alternative (Results 3)
i.e. (-R3 alternative – (-R2 null) = R2 null – R3 alternative.

The Likelihood Ratio Test (LRT) for haplotype-phenotype association adjusted for covariates is then
χ^2 **= 2 x ((-R3 alternative – (-R2 null)) with (df alternative – df null) df**

Find *P* from chi sq tables.

Testing successive phenotype variables
Return to the beginning of the Results 2 procedure.
Select the next phenotype in the LH panel.
Deselect all the haplotypes in the RH panel, except the first reference haplotype.
Deselect "Estimation of haplotype effects".
Run Results 2 for the second phenotype, followed by Results 3.

Chapter 3: CRESSIDA study – effect of integrated dietary intervention on plasma and erythrocyte fatty acid composition

3.1 Introduction

Recommendations for the prevention of CVD have been based on epidemiological evidence suggesting a relationship between certain dietary components and risk of CVD and the effects of these components on surrogate markers of risk. Most attention has been focused on dietary fat and salt but more recently has broadened to include increased intakes of fruit and vegetables, fish and whole grains and reduced intake of added sugars and high glycaemic index carbohydrate (CHO) foods. Most dietary intervention trials have focused on modifying single components of diet and few have modified more than one component. The DASH study was the first large scale study to evaluate the effects of multiple dietary interventions on blood pressure. This study showed that the effects on blood pressure of dietary advice to increase fruit and vegetable intake was enhanced when accompanied by advice to increase the intake of low fat dairy products and whole grains (Appel *et al*. 2006).

Serum total cholesterol (TC) is positively associated with CVD risk (Lewington *et al*. 2007), which increases with age and BMI. Dietary intervention studies show that the intake of C12-C16 SFA increase TC and LDL-C concentrations compared with unsaturated fatty acids or CHO (Mensink *et al*. 2003). However, prospective studies have been unable to demonstrate an effect of SFA intake on serum cholesterol concentrations. Keys & Parlin (1966) constructed an equation to predict the effect of increasing intake of C12-C16 SFA on serum cholesterol concentrations in mmol/L shown below.

$$\Delta cholesterol \ \text{(mmol/L)} = \frac{(2.3\Delta SFA\ \%energy - \Delta PUFA\ \%\ energy)}{38.5}$$

The limitation of the Keys equations is that they were based on dietary intakes of 40% energy of fat. The equations developed by Mensink *et al.* (2003) were elaborated to compare changes in TC, LDL-C, HDL-C, TAG and the ratio of TC:HDL-C compared with CHO, MUFA, *trans* fatty acids (TFA) and PUFA. However, these do not include the effects of long-chain *n*-3 PUFA on serum lipids. The consumption of high amounts of these fatty acids was reviewed by Balk *et al*. (2006) and show that a reduction in plasma TAG. However, the amounts provided by consuming intakes equivalent to two portions of fish a week are more modest (Sanders *et al*. 2006a; Sanders *et al*. 2011) and generally do not affect TC or LDL-C concentrations. Plasma and erythrocyte fatty acid composition is very sensitive to small increases in the intake of LC *n*-3 PUFA. The aim of this chapter was to see if was possible to develop a global biomarker of compliance to dietary fat modification that would indicate compliance with the dietary fat targets for a cardio-protective diet.

The measurement of carotid intimal media thickness (CIMT) by high resolution ultrasound is a non-invasive method to gauge the extent of atherosclerosis of the carotid artery in health. The MESA study reported an association between the omega-3 index and CIMT (He *et al*. 2009). Data were available on CIMT on the participants of the CRESSIDA study and it was decided, therefore, to conduct exploratory analyses to see if there was any relationship between CIMT in the study population and fatty acid composition of blood biomarkers or omega-3 fatty acid intake at baseline.

3.2 Purpose of the study

Current dietary fat advice for the prevention of cardiovascular disease (CVD) in the United Kingdom is based on the recommendations of the 1994 COMA report. It recommends a reduction in SFA intake and their partial replacement with MUFA, no increase in *n*-6 PUFA but an increase in *n*-3 PUFA from increased consumption of oily fish. In order to assess the use of plasma and erythrocyte lipids to ascertain compliance to such advice, analyses of erythrocyte and plasma lipids were compared in subjects participating in a randomized controlled trial of diet (CRESSIDA).

The CRESSIDA trial was a multifaceted dietary intervention trial whose targets involved modification of fat intake, reduction of salt intake, increased consumption of fruits and vegetables, increased intake of wholegrain cereals (50% of the intake) and a restricted intake of added sugars. The study aimed to recruit 196 subjects who were randomized to either a control diet, which represented the typical British diet, or an intervention diet that approximated to the dietary recommendations of COMA (1994).

Thus the objectives of this chapter were:

1. To analyse erythrocyte phospholipids and total plasma fatty acid composition by gas chromatography.
2. To evaluate the use of these biomarkers as an indicator of compliance to dietary fat recommendations.

3. To conduct exploratory analysis to examine whether the fatty acid biomarkers were related to the extent of carotid intimal medial thickening at baseline.

Hypothesis

Alterations in the fatty acid composition of plasma and erythrocyte lipids will indicate compliance to the intervention diet.

3.3 Study design and dietary intervention

3.3.1 Study design

The CRESSIDA study was a parallel randomized controlled trial design on risk factors of CVD and vascular function in non-smoking men and women aged 40-70 y, who as a consequence of their age are at higher risk of CVD than younger people. The study aimed to compare on CVD risk factors the influence of implementation of an integrated dietary approach versus a habitual UK diet (control diet). The intervention diet was restricted to a low salt (<6 g/d) and SFA intake (<10% energy), increased intake of whole grain and cereals (>50%), fruit and vegetables (5 portions/week) and consumption of oily fish (2 portions/week one of which should be oily).

The primary outcome was a change in major cardiovascular risk factors, which were defined as 4 mmHg change in day-time blood pressure and a 5% change in TC:HDL ratio. Secondary outcomes were changes in endothelial function, arterial stiffness, insulin sensitivity and C-reactive protein concentrations. However, the focus of this Chapter is on indices of compliance to the dietary fat advice. **Figure 3.1** shows the overall study design.

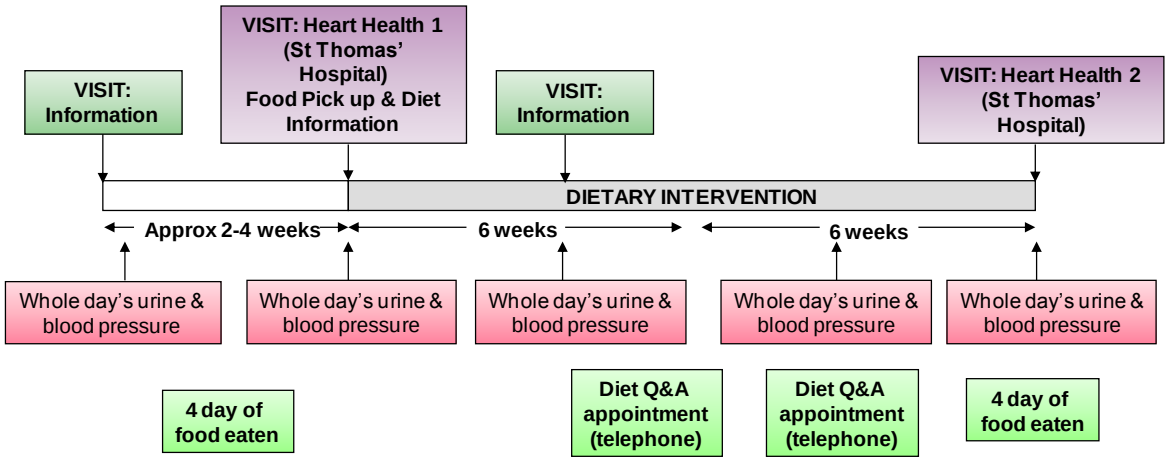


Figure 3.1 Overall design of the CRESSIDA study

3.3.2 Dietary intervention

Dietary fat formulation approach was a modified dietary fat exchange model. Participants were provided with vegetable oils and spreading fats and dietary advice regarding type of milk products and choice and preparation of meat. **Table 3.1** summarises dietary intakes targets.

Table 3.1 Composition of control and intervention diets

	Control diet	Intervention diet
SFA	~ 14 % en	~ 10 % en
MUFA	~12 % en	~16 % en
PUFA	~6 % en PUFA	~6 % en
TFA	<1 % en TFA	< 1% en
Salt	No restrictions	<6 g/d
Oily fish	<1 serving/month	2 portion/week
Fruit & veg/day	3 portions	5 portions
Wholegrain cereals	-	>50% en

% en=% food energy

3.4 Study participants

Study participants were healthy non-smoking men and women aged 40-70 y with a systolic BP <160 mmHg and diastolic BP <105 mmHg and were not receiving medication for treatment of high blood pressure or taking lipid-lowering medications. Participants were recruited by newspaper advertisement. Randomization to treatment was conducted by minimization stratifying for age, gender, ethnicity and BMI using an

online electronic clinical trial database (MedSciNet AB, Stockholm, Sweden). Where two participants cohabited, both were allocated to the same treatment group.

Participants were required to complete 4-day diet records at baseline and at follow-up. The records were checked by a dietician (Dianne Reidlinger) and coded into the dietary analysis program NETWISP (version 3.0). Basal metabolic rate was estimated using the Schofield equations as described in the Dietary Reference Values report (1991) and participants with energy intake less than 1.2 x BMR were regarded as under-reporters and were excluded as a result.

3.5 Analysis of fatty acid proportions and fasting blood lipids

Erythrocyte lipid extracts were prepared and analysed by Robert Gray (Diabetes and Nutritional Sciences Division, King's College London) using methods as previously described in Chapter 2 and plasma fatty acids were prepared and analysed by author. The fasting lipid profile was determined by Tracy Dew at KingsPath at King's College London under the supervision of Dr Roy Sherwood. TC, HDL-C and TAG concentrations were measured using fully enzymatic procedures on the Siemens ADVIA 2400 automated chemistry analyser. LDL-C was estimated by the Friedwald equation as described in Chapter 2. Carotid intima-medial thickness (CIMT) assessed by high resolution ultrasound. CIMT was measured as described in Chapter 2 on all

participants at the end of the study by a trained ultrasonographer Dr Benyu Jiang at St Thomas' Hospital.

3.6 Statistical analysis

Statistical analysis was conducted using IBM SPSS/PC (version 20). Standard distributional checks were made on all data and where data did not conform to normal distribution a $\log_e$ transformation was attempted. Comparison between treatments were analysed by analysis of covariance (ANCOVA) adjusting for covariates used in the minimisation procedure (age, gender, ethnicity and body mass index). Spearman's correlation was performed between individual fatty acids of erythrocyte phospholipids and total plasma fatty acids. Data reduction conducted using principal component analysis (PCA). Obtained components were then subjected to varimax rotation to calculate orthogonal solutions.

3.7 Results

3.7.1 Characteristics of the study population

A total of 162 participants completed the study (3-months) and their characteristics are shown in **Table 3.2**. BMI at baseline was 1.5 kg/m^2 lower in the control group but otherwise the participants well matched.

Table 3.2 Baseline characteristics of the study participants stratified by group.

	Control (*n*= 82)	Intervention (*n*= 80)
Male	32 (39%)	32 (40%)
Female	50 (61%)	48 (60%)
Age (y)	52.4±8.1	52.8±7.9
BMI (kg/m^2)[1]	26.9±3.9	25.4±3.7*
Systolic BP (mm Hg)	119.7±14.8	120.2±17.1
Diastolic BP (mm Hg)	79.4±10.0	78.1±9.1
Heart rate (beats/min)	67.6±9.3	65.3±8.8
Glucose (mmol/L)	5.2±0.4	5.3±0.5
Insulin (mU/L)	8.4±6.3	6.6±4.2
HsCRP (mg/dL)	1.7±2.0	1.8±3.0
Triacylglycerol (mmol/L)	1.3±0.6	1.2±0.6
Cholesterol (mmol/L)	5.1±1.0	5.1±1.2
LDL-cholesterol (mmol/L)	3.1±0.8	3.0±0.9
HDL-cholesterol (mmol/L)	1.5±0.4	1.5±0.4
TC:HDL-C ratio	3.6±1.0	3.5±0.9

Mean values with SD. *P=0.03 compared with control, multivariate analysis adjusted for gender.

3.7.2 Dietary intake of the study participants

The effects of the dietary intervention on the dietary intakes are presented in **Table 3.3**. Protein as a proportion of the dietary energy was slightly greater in intervention group. There were no differences in carbohydrates intake, but the intakes of fat and SFA were lower in the intervention group, and those of MUFA and PUFA were greater. The estimated intake of LA was greater following the intervention on the integrated dietary approach compared with the control diet, as were the intakes of EPA and DHA. Excluding data from participants with energy intakes <1.2 x BMR did not alter these findings (12 in the intervention group and 9 in the control).

Table 3.3 Estimated nutrient intake from 4-day food diaries completed at baseline and at endpoint

	Intervention[1]	Control[2]	Difference[3]	*P*
Energy (MJ/day)				
Baseline	8.99 (2.26)	8.95 (2.48)		
Endpoint	8.73 (2.31)	9.35 (2.34)	-0.62 (-1.35, 0.10)	0.09
Protein (% energy)				
Baseline	16.0 (2.9)	15.8 (3.2)		
Endpoint	17.7 (2.9)	15.7 (2.9)	1.8(-0.2, 3.8)	<0.001
CHO (% energy)				
Baseline	47.0 (7.4)	48.8 (7.5)		
Endpoint	47.1 (7.0)	46.7 (6.4)	0.3 (-4.5, 5.0)	0.158
Fat (% energy)				
Baseline	36.1 (5.9)	34.7 (6.2)		
Endpoint	33.8 (5.4)	37.1 (5.3)	-3.6 (-5.2, -1.9)	<0.001
SFA (% energy)				
Baseline	12.3 (3.5)	11.6 (3.2)		
Endpoint	7.7 (1.7)	14.8 (2.8)	-7.2 (-8.0, -6.5)	<0.001
MUFA (% energy)				
Baseline	11.9 (2.5)	11.8 (2.7)		
Endpoint	15.7 (3.5)	12.3 (2.1)	3.4 (2.5, 4.5)	<0.001
PUFA (% en				
Baseline	6.6 (2.2)	6.1 (1.7)		
Endpoint	7.0 (1.8)	5.3 (1.7)	1.9 (1.4, 2.4)	<0.001
Linoleic acid (% energy)				
Baseline	6.2 (2.1)	5.7 (1.6)		
Endpoint	6.3 (1.5)	4.9 (1.6)	1.4 (0.9, 1.9)	<0.001
n-3 PUFA (g/d)				
Baseline	0.23 (0.47)	0.36 (0.82)		
Endpoint	1.39 (1.66)	0.08(0.15)	1.32 (0.95, 1.32)	<0.001
EPA (g/d)				
Baseline	0.07 (0.16)	0.11 (0.25)		
Endpoint	0.42 (0.57)	0.02 (0.09)	0.39 (0.27, 0.52)	<0.001
DHA (g/d)				
Baseline	0.13 (0.28)	0.21 (0.48)		
Endpoint	0.82 (0.95)	0.04 (0.09)	0.77 (0.57, 0.99)	<0.001

[1] *n*= 80,[2] *n*= 82, [3] mean difference with 95% CI.

Mean values (SD).

Comparisons on treatment are by multivariate analysis adjusted by gender.

3.7.3 Changes in serum lipid concentrations

Table 3.4 shows the serum lipid concentrations. The intervention diet resulted in an 8.1% fall in serum cholesterol and 10% fall in LDL-C compared with the control treatment. However, HDL-C differed by 6.2% from the control value. Overall, the TC:HDL-C ratio was 4.2% lower on intervention diet compared with the control diet and serum TAG concentration was 8.8% lower. The change in TC between diets was very close to that predicted by the Keys equation (Δ cholesterol (mg/dL) = 2.3 ΔS –ΔP) which can be divided by 38.5 convert to mmol/L (ΔS is the difference in SFA % energy minus stearic acid and ΔP is the difference in energy % from PUFA). Substituting ΔS as 6.0 and ΔP as 1.9 predicts a 0.41 mmol/L difference in TC. The difference in cholesterol intake would be predicted to result in a 0.06 mmol/L difference between diets. The observed difference in serum cholesterol between diets was 0.46 mmol/L *vs.* the estimated 0.47 mmol/L. The results of the dietary analysis and the changes in serum cholesterol concentration indicate that the participants were compliant with the dietary advice given. The change in serum TAG concentrations is close to what would be predicted from the results of the MARINA and OPTILIP studies for this level of intake of *n*-3 LC-PUFA.

Table 3.4 Changes in serum lipid concentrations following control or intervention diet

		Intervention[1]	**Control**[2]	**Treatment effect** [3]	***P***[4]
		n= 80	*n*= 82		
Cholesterol[5]	Baseline	5.33 (1.11)	5.35 (0.86)		
(mmol/L)	Endpoint	5.06 (0.93)	5.49 (0.89)	-8.1% (-11.4, -4.8)	<0.0001
LDL-C	Baseline	3.18 (0.89)	3.18 (0.77)		
(mmol/L)	Endpoint	3.00 (0.75)	3.29 (0.78)	-10.0% (-14.3, -5.7)	<0.0001
HDL-C[5]	Baseline	1.61 (0.40)	1.56 (0.42)		
(mmol/L)	Endpoint	1.58 (0.38)	1.62 (0.44)	-6.2 % (-10.3, -2.0)	0.005
TC:HDL-C[5]	Baseline	3.46 (0.91)	3.63 (0.98)		
	Endpoint	3.31 (0.87)	3.59 (0.96)	-4.2% (-8.0, 0.2)	0.044
TAG[5]	Baseline	1.24 (0.59)	1.33 (0.58)		
(mmol/L)	Endpoint	1.06 (0.45)	1.23 (0.55)	-8.8 % (-15.9, -1.1)	0.027

Mean values (SD).

[1]*n*= 80, [2]*n*= 82.

[3]Percentage change (95% CI) on treatment adjusted for age, BMI, ethnicity, gender and baseline value.

[4]Probability of treatment effect from analysis of covariance regressed against age, BMI, ethnicity, gender and baseline value

3.7.4 Erythrocyte phospholipids and total plasma fatty acid composition pre- and post-intervention

Table 3.6 and **Table 3.7** show the changes in proportions of fatty acids in erythrocyte phospholipids and total plasma lipids in control and intervention groups respectively. There were no significant differences between groups in fatty acid proportions at baseline. The most abundant fatty acids in erythrocytes membrane were 16:0, 20:4*n-6*, 18:1*n-9* and 18:0. While the plasma was associated with higher levels of 18:2*n-6*, 18:1*n-9*, 16:0, 20:4*n-6* and 18:0. A significant amount of DHA (22:6*n-3*) was found in erythrocyte membrane (5.69%) in comparison with plasma (2.35%). EPA (20:5*n-3*) was similar in the two biomarkers. Alpha-linolenic (ALA, 18:3*n-3*) and palmitoleic acids (16:1*n-7*) were both lower in erythrocytes. Proportions of 18:1*trans* isomers were low but were slightly greater in erythrocytes.

Significant differences between treatments were observed following intervention in the proportions in erythrocyte lipids of 20:5*n-3* ($P<0.001$), 22:6*n-3* ($P<0.001$), 18:1*n-9* ($P=0.008$) and MUFA ($P=0.004$) whereas proportions of 22:5*n-6* ($P=0.002$), 20:3*n-6* ($P=0.008$), 20:4*n-6* ($P=0.018$), 18:2*n-6* ($P=0.02$), and 22:4*n-6* ($P=0.037$) were correspondingly lower compared with the control group. No other significant differences were noted.

Significant differences between treatments were observed following intervention in plasma proportions of 20:5*n-3* ($P<0.001$), 22:6*n-3* ($P<0.001$), 18:*1n-9* ($P=0.002$), 22:5*n-6* ($P=0.006$), 22:5*n-3* ($P=0.012$), PUFA ($P=0.018$) and MUFA ($P=0.028$) were

increased, whereas the proportions of SFA (P<0.001), 14:0 (P<0.001), 16:0 (P<0.017), 18:0 (P<0.001), 18:3n-6 (P<0.001), 20:3n-6 (P<0.001), 16:1*n-7* (P=0.001), 18:3*n-3* (P=0.001), 20:4*n-6* (P=0.004) and 16:0 (P=0.017) fell compared with the control group. No other significant differences were noted.

Lower levels of GLA, DGLA and AA in the intervention group might indicate inhibition of D6D by LC-PUFA. The proportion of osbond acid appeared high in plasma but not in erythrocyte lipids. It is possible that this may reflect co-elution of 24:2*n*-6 with 22:5*n*-6. The slightly high proportion of ALA in the plasma of the control group might be due to higher intake of dairy fat and red meat (beef, lamb) in the control, which contains appreciable amount of ALA (1-2% by wt). Increased proportions of EPA, DPA and DHA indicate compliance to dietary advice to consume oily fish.

Receiver operator curves (ROC) were plotted to estimate the sensitivity of total plasma SFA as a predictor of a diet high in SFA. **Figure 3.2** shows receiver operator curves for proportion of total SFA in plasma and erythrocyte lipids to identify subjects on high SFA diet *vs*. intervention diet. Area under the curve was 0.72 (95 % CI 0.62, 0.80) for plasma and 0.47 (95% CI 0.38, 0.56) for erythrocyte as summarised in **Table 3.5**.

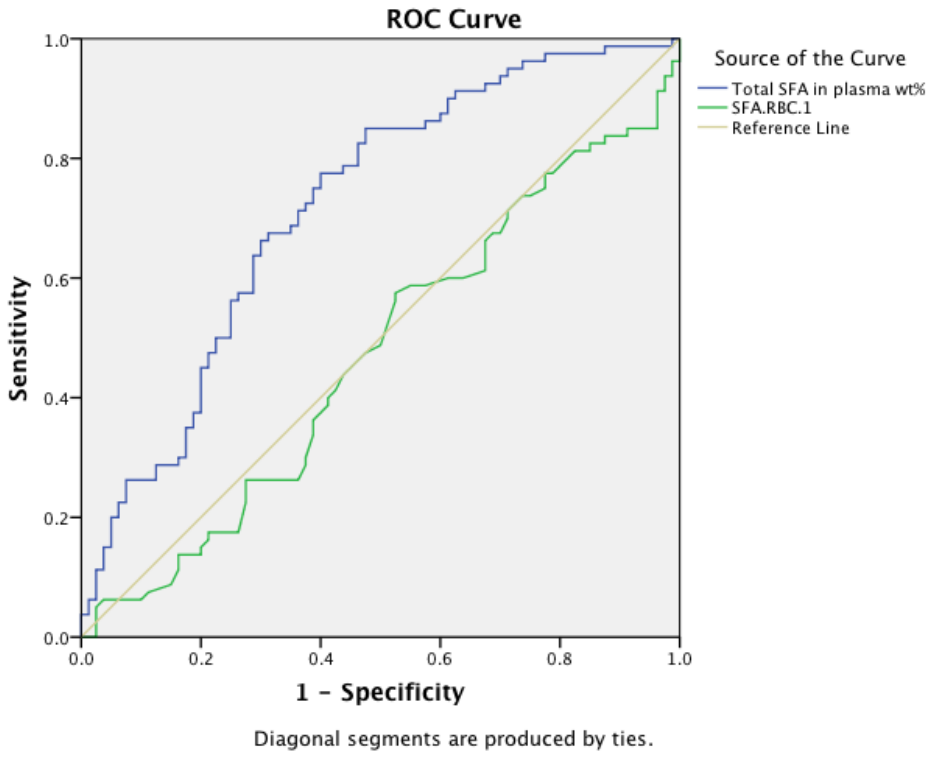


Figure 3.2 Receiver operator curves for proportion of total SFA in plasma and erythrocyte lipids to identify subjects on high SFA diet *vs* intervention diet.

Table 3.5 Area under the curve for the proportions of total SFA in plasma and erythrocyte lipids.

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
SFA in plasma wt%	0.719	0.040	0.000	0.640	0.798
SFA in RBC wt%	0.470	0.046	0.518	0.381	0.560

The test result variable(s): Total SFA in RBC wt% has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

[a]Under the nonparametric assumption

[b]Null hypothesis: true area = 0.5

Table 3.6 The proportions of saturated (SFA), trans (TFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in erythrocyte lipids at baseline and after 3-months on control or intervention diet.

Fatty Acid	Baseline (*n*= 162)	Control (*n*= 82)	Intervention (*n*= 80)	Mean difference	*P*
SFA	35.40 ± 3.62	35.01 (34.22,35.79)	34.47 (33.69,35.25)	-0.53 (-1.66,0.59)	0.350
16:0	19.86± 2.87	19.76 (19.18,20.35)	19.52 (18.94,20.10)	-0.24 (-1.08,0.59)	0.819
18:0	15.54± 1.78	15.27 (14.82,15.73)	14.92 (14.47,15.37)	-0.35 (-1.00,0.30)	0.486
MUFA	17.31± 1.43	17.60 (17.31,17.89)	18.22 (17.92,18.52)	0.62 (0.20,1.04)	0.004
16:1*n-7*	0.43± 0.24	0.41 (0.36,0.45)	0.42 (0.38,0.46)	0.02 (-0.04,0.08)	0.614
18:1*n-9*	15.78± 1.30	15.86 (15.46,16.26)	16.64 (16.24,17.04)	0.78 (0.21,1.35)	0.008
18:1*n-7*	1.12 ± 0.41	1.31 (1.02,1.60)	1.25 (0.96,1.55)	-0.06 (-0.47,0.36)	0.726
TFA: 18:1 *trans*	0.25± 0.35	0.19 (0.16,0.22)	0.16 (0.12,0.19)	-0.03 (-0.08,0.01)	0.343
PUFA	43.14± 3.85	43.32 (42.48,44.16)	43.42 (42.57,44.26)	0.10 (-1.10,1.29)	0.873
18:2*n-6*	11.64 ± 1.63	11.67 (11.39,11.94)	11.20 (10.92,11.47)	-0.47 (-0.87,-0.08)	0.020
20:3*n-6*	1.93± 0.42	1.93 (1.86,1.99)	1.80 (1.74,1.87)	-0.13 (-0.22,-0.03)	0.008
20:4*n-6*	15.87± 2.08	16.01 (15.56,16.46)	15.22 (14.77,15.67)	-0.78 (-1.43,-0.14)	0.018
22:4*n-6*	2.79± 0.78	2.81 (2.66,2.96)	2.58 (2.43,2.73)	-0.23 (-0.44,-0.01)	0.037
22:5*n-6*	0.42± 0.16	0.45 (0.42,0.48)	0.38 (0.35,0.41)	-0.07 (-0.12,-0.03)	0.001
18:3*n-3*	0.26± 0.27	0.34 (0.26,0.42)	0.29 (0.21,0.36)	-0.05 (-0.16,0.06)	0.332
20:5*n-3*	1.41± 0.74	1.21 (1.09,1.33)	1.83 (1.71,1.95)	0.62 (0.45,0.79)	<0.001
22:5*n-3*	3.06± 0.65	3.19 (2.97,3.41)	3.07 (2.85,3.29)	-0.12 (-0.43,0.19)	0.438
22:6*n-3*	5.69± 1.71	5.26 (4.94,5.58)	6.50 (6.18,6.82)	1.25 (0.78,1.71)	<0.001
n-6:*n-3*	3.40± 0.96	3.46 (3.28,3.64)	2.79 (2.61,2.98)	-0.66 (-0.92,-0.40)	<0.001

Baseline data presented as mean ± SD; no differences between groups as baseline.

P-value for difference between control and intervention groups adjusted for baseline value, age, gender, ethnicity and BMI.

Table 3.7 Saturated (SFA), trans (TFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids (wt%) in total plasma lipids at baseline and at follow-up.

Fatty Acid	Baseline (*n*= 162)	Control (*n*= 82)	Intervention (*n*= 80)	Mean difference	*P*
SFA	27.94 ± 1.94	27.93 (27.68,28.18)	27.01 (26.76,27.26)	-0.92 (-1.28,-0.56)	<0.001
14:0	0.84 ± 0.30	0.83 (0.78,0.88)	0.66 (0.61,0.71)	-0.17 (-0.24,-0.11)	<0.001
16:0	20.32 ± 1.75	20.23 (19.99,20.47)	19.81 (19.56,20.05)	-0.42 (-0.77,-0.08)	0.017
18:0	6.50 ± 0.57	6.59 (6.50,6.68)	6.29 (6.20,6.39)	-0.30 (-0.43,-0.17)	<0.001
20:0	0.28 ± 0.11	0.28 (0.26,0.29)	0.26 (0.24,0.27)	-0.02 (-0.04,0.00)	0.036
MUFA	24.90 ± 3.39	24.58 (24.15,25.01)	25.28 (24.84,25.71)	0.69 (0.08,1.31)	0.028
16:1n-7	2.34 ± 0.97	2.28 (2.17,2.38)	2.02 (1.91,2.12)	-0.26 (-0.41,-0.11)	0.001
18:1n-9	20.85 ± 2.75	20.64 (20.26,21.01)	21.52 (21.13,21.90)	0.88 (0.34,1.42)	0.002
18:1n-7	1.71 ± 0.35	1.67 (1.63,1.72)	1.74 (1.70,1.78)	0.06 (0.00,0.12)	0.035
TFA 18:1	0.13 ± 0.16	0.13(0.07,0.18)	0.12(0.07,0.18)	0.10 (-0.05,0.25)	0.209
PUFA	42.47 ± 4.12	42.68 (42.14,43.23)	43.63 (43.08,44.19)	0.95 (0.17,1.74)	0.018
18:2*n*-6	27.31 ± 3.84	27.56 (27.10,28.01)	27.62 (27.16,28.09)	0.07 (-0.59,0.73)	0.845
18:3*n*-6	0.50 ± 0.19	0.51 (0.48,0.53)	0.43 (0.40,0.45)	-0.08 (-0.12,-0.04)	<0.001
20:3*n*-6	1.57 ± 0.37	1.64 (1.58,1.70)	1.43 (1.37,1.49)	-0.21 (-0.30,-0.13)	<0.001
20:4*n*-6	6.58 ± 1.44	6.74 (6.56,6.93)	6.35 (6.17,6.54)	-0.39 (-0.65,-0.13)	0.004
22:4*n*-6	0.44 ± 0.19	0.46 (0.45,0.48)	0.46 (0.44,0.47)	-0.01 (-0.03,0.01)	0.517
22:5*n*-6	0.83 ± 0.33	0.85 (0.81,0.89)	0.92 (0.89,0.96)	0.07 (0.02,0.13)	0.006
18:3*n*-3	0.68 ± 0.20	0.67 (0.63,0.70)	0.59 (0.56,0.62)	-0.07 (-0.12,-0.03)	0.001
20:5*n*-3	1.34 ± 0.88	1.22 (1.04,1.40)	2.02 (1.84,2.19)	0.79 (0.54,1.05)	<0.001
22:5*n*-3	0.86 ± 0.65	0.84 (0.76,0.93)	1.00 (0.91,1.08)	0.16 (0.03,0.27)	0.012
22:6*n*-3	2.35 ± 1.13	2.18 (2.04,2.31)	2.83 (2.70,2.97)	0.66 (0.47,0.85)	<0.001
n-6:*n*-3 ratio	7.77 ± 2.20	8.23 (7.84,8.61)	6.34 (5.96,6.73)	-1.88 (-2.43,-1.33)	<0.001

Mean ± SD or (95% CI). *P*-value for difference adjusted for baseline value, age, gender, ethnicity and BMI.

3.7.5 Correlations between erythrocyte lipids and plasma total fatty acid composition post intervention.

Spearman's correlations between erythrocyte lipid and total plasma fatty acid composition are shown in **Table 3.8**. Strong correlations were found for 20:5*n-3* (ρ=0.725), *n-6*:*n-3* ratio (ρ= 0.654; *P*< 0.001); 20:3*n-6* (ρ= 0.616), 18:2*n-6* (ρ= 0.537), 16:1*n-7* (ρ= 0.522), 22:6*n-3* (ρ= 0.506), 20:4*n-6* (ρ= 0.380), 18:1*n-7* (ρ= 0.347), 22:5*n-3* (ρ= 0.338) and TFA (ρ= 0.305). Weaker correlation were found for MUFA (ρ= 0.236), 16:0 (ρ= 0.197, *P*= 0.012), 18:1*n-9* (ρ= 0.193, *P*= 0.014), SFA (ρ= 0.190, *P*= 0.016), and 18:0 (ρ= 0.179, *P*= 0.023). These findings indicate that erythrocyte lipids are good indices of the balance between *n*-6 and *n-3* LC-PUFA and a low intake of PUFA as judged by lower proportions of LA and high proportions of palmitoleic acid which were negatively correlated (ρ= -0.207, *P*= 0.009) but are not a good indicator of the PUFA/SFA ratio. This is not entirely surprising as there is specificity in phosphatidyl choline for palmitic acid in the *sn*-1 position and for stearic acid in ethanolamine and serine phosphoglycerides which are the major phosphoglycerides in erythrocyte lipids. In plasma, cholesteryl esters have high specificity for LA and choline phosphoglycerides, which make up the bulk of plasma phospholipids, have a high content of palmitic acid in the *sn*-1 position and a high specificity for LA in the *sn*-2 position. Plasma TAG concentrations are more likely to reflect dietary fat composition or that remodelled in the liver.

Table 3.8 Correlations between proportions of fatty acid in plasma and erythrocyte lipids after 3 months on the intervention or control diet.

Fatty Acid	ρ	*P*
SFA	0.190	0.016
16:0	0.197	0.012
18:0	0.179	0.023
MUFA	0.236	
16:1*n-7*	0.522	<0.001
18:1*n-9*	0.193	0.014
18:1*n-7*	0.347	<0.001
TFA		
18:1 *trans*	0.305	<0.001
n-6		
18:2*n-6*	0.537	<0.001
20:3*n-6*	0.616	<0.001
20:4*n-6*	0.380	<0.001
22:4*n-6*	-0.228	0.004
22:5*n-6*	-0.191	0.019
n-3		
18:3*n-3*	0.121	0.141
20:5*n-3*	0.72	<0.001
22:5*n-3*	0.338	<0.001
22:6*n-3*	0.506	<0.001
n-6:*n-3* ratio	0.654	<0.001
P:S ratio	0.095	0.267

ρ denotes Spearman's correlation. *n*= 162

3.7.6 Principal component analysis

Data reduction was attempted using principal component analysis on the plasma and erythrocyte fatty acids post intervention. **Table 3.9** and **Table 3.10** show the components for the analysis and **Figure 3.3** and **Figure 3.4** show the scree plots generated from plasma and erythrocyte phosphoglycerides respectively. **Table 3.11** and **Table 3.12** show the differences between control and intervention groups for the components. Component 1, which explained 24% of the variance, appeared to reflect changes in the P/S ratio and is being determined by higher proportions of SFA and MUFA, a lower proportion of LA and higher proportion of LC-PUFA. This is likely to indicate a low absolute intake of PUFA which favours desaturation and chain elongation of the parent fatty acids into longer chain derivatives as well as enhanced desaturation of palmitic to palmitoleic and stearic to oleic acids. Component 2, which explained 14.1% of the variance showed a strong correlation with arachidic, adrenic, osbond acid and DHA and may indicate a higher intake of LC-PUFA. Component 3 explained 11.8% of the variance and was associated with higher proportions of EPA and 22:5*n-3* but lower proportions of the products of desaturation of LA, GLA and DGLA. Component 4 was found to explain 9.1% of the variance and correlates well with myristic, oleic, ALA and 22:5*n-3* and negatively correlated with AA and DHA. When looking at component 5, it appeared to explain 8.1% of the variability and was well correlated with stearic, GLA, DGLA, AA and 22:5*n-3*. It was also found to correlate negatively with MUFA and DHA. Component 6 was found to explain as little as 5.7%

of the variability and correlates strongly with vaccinic, AA, 22:5*n-3* and negatively with TFA and DHA.

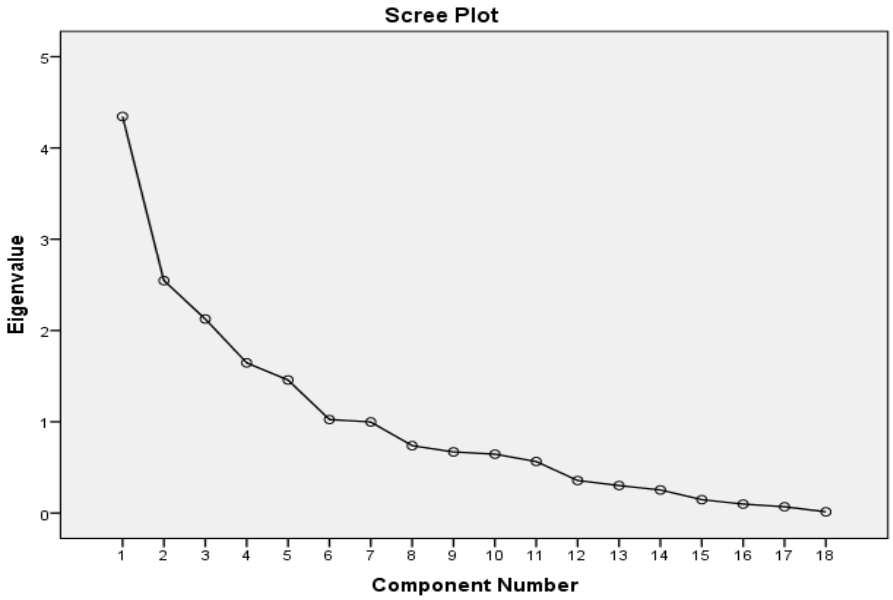


Figure 3.3 Scree plot of the components generated from the application of principal component analysis on the 18 plasma fatty acids.

Table 3.9 Association between 18 plasma fatty acid proportions and the 6 components identified in the CRESSIDA study following 3-months intervention.

Fatty Acid	1	2	3	4	5	6
	PS ratio	Low MUFA	Increased n-3 LCP	Low *n*-6 high *n*-3	Increased ALA, DHLA AA	Low SFA
Variance explained (%)	24.1	14.1	11.8	9.1	8.1	5.7
Eigen value	4.3	2.5	2.1	1.6	1.5	1.0
SFA						
14:0	**0.606**	**-0.284**	-0.145	**0.517**	0.153	-0.047
16:0	**0.790**	0.070	0.004	0.053	-0.068	0.042
18:0	**-0.211**	0.021	-0.043	-0.086	**0.718**	-0.071
20:0	0.1017	**0.8824**	-0.1050	0.0261	-0.125	0.027
MUFA						
16:1*n-7*	**0.745**	0.015	-0.130	0.082	**-0.304**	0.155
18:1*n-9*	**0.292**	-0.160	**-0.389**	**0.255**	**-0.570**	-0.139
18:1*n-7*	**0.322**	**-0.284**	0.166	0.102	**-0.407**	**0.563**
TFA						
18:1 *trans*	0.084	-0.023	0.146	0.015	-0.021	**-0.677**
PUFA						
n-6						
18:2*n-6*	**-0.884**	0.080	-0.095	0.133	0.165	0.165
18:3*n-6*	**0.519**	**-0.351**	**-0.332**	-0.053	**0.538**	0.061
20:3*n-6*	**0.503**	-0.056	**-0.595**	0.003	**0.254**	0.124
20:4*n-6*	0.098	-0.104	-0.036	**-0.735**	**0.392**	**0.201**
22:4*n-6*	-0.073	**0.916**	-0.068	-0.144	0.134	-0.075
22:5*n-6*	-0.133	**0.864**	**0.267**	-0.119	0.040	-0.098
n-3						
18:3*n-3*	0.012	-0.097	-0.009	**0.675**	-0.113	0.093
20:5*n-3*	0.047	0.047	**0.881**	-0.111	-0.005	-0.189
22:5*n-3*	0.067	**-0.264**	**0.593**	**0.439**	**0.282**	**0.394**
22:6*n-3*	-0.188	**0.230**	0.193	**-0.575**	**-0.312**	**-0.450**

Bold indicates strong correlation

Components generated from PCA analysis from erythrocyte phospholipids are summarised in **Table 3.10. Figure 3.4** shows scree plot generated from the application of PCA on the erythrocytes phospholipids. Component 1, which explained 19.6% of the variability, correlates strongly with *n-6* PUFA with the exception of LA. It correlates negatively with palmitic, EPA and DHA. Component 2 explained 18% of the variability and was found to correlate strongly with palmitic, vaccinic, and osbond acids and negatively with oleic acid. Component 3 explained 13% of the variability and strongly correlated with AA, adrenic acids and *n*-3 PUFA with the exception of ALA. It was negatively correlated with palmitic, stearic, palmitoleic and ALA. Component 4 explained 10.2% of the variability and correlates with stearic and *n-3* PUFA with the exception of ALA and EPA. It correlates negatively with the essential fatty acids LA and ALA. Finally component 5, which explained 7.3% of the variability, correlated strongly with stearic, TFA, LA and AA. It also correlated negatively with palmitoleic, DGLA and 22:5*n-3*.

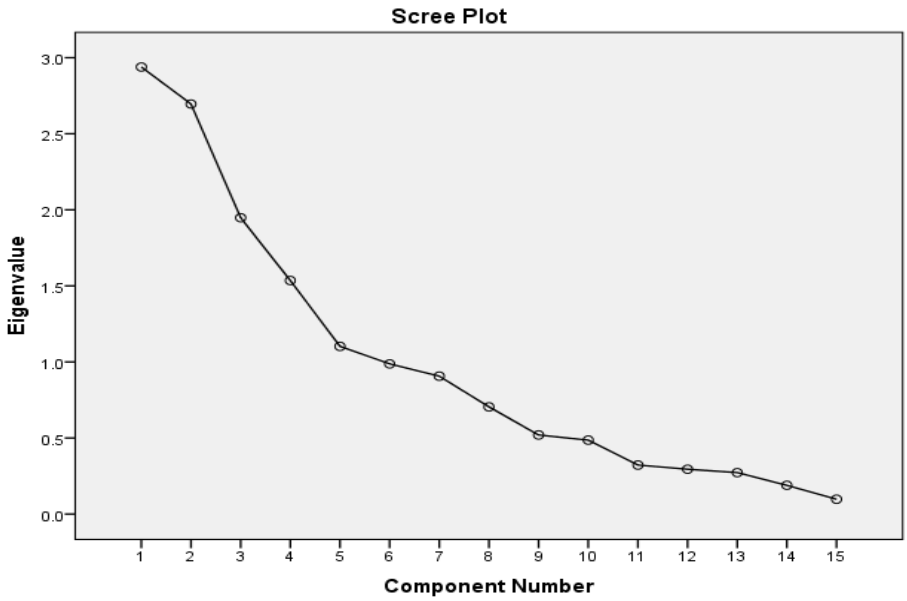


Figure 3.4 Scree plot of the components generated from the application of principal component analysis on the 15 RBC fatty acids.

Table 3.10 Association between 15 fatty acid proportions in erythrocytes and the 5 components identified in the CRESSIDA study following 3-months intervention.

Fatty Acid	1	2	3	4	5
	n-6:n-3	High SFA, low MUFA	High LCP	Low EFA	High TFA
Variance explained (%)	19.6	18.0	13.0	10.2	7.3
Eigen value	2.9	2.7	1.9	1.5	1.1
SFA					
16:0	**-0.272**	**0.215**	**-0.814**	0.002	-0.142
18:0	0.112	-0.072	**-0.216**	**0.725**	**0.280**
MUFA					
16:1*n-7*	0.099	-0.178	**-0.449**	0.135	**-0.665**
18:1*n-9*	-0.019	**-0.892**	0.016	-0.061	-0.050
18:1*n-7*	-0.148	**0.868**	-0.129	-0.037	-0.044
TFA					
18:1 *trans*	0.048	-0.026	-0.120	0.174	**0.578**
PUFA					
n-6					
18:2*n-6*	-0.047	-0.077	-0.109	**-0.672**	**0.336**
20:3*n-6*	**0.599**	0.053	0.120	0.190	**-0.206**
20:4*n-6*	**0.556**	0.067	**0.485**	-0.058	**0.255**
22:4*n-6*	**0.803**	-0.152	**0.335**	0.191	0.103
22:5*n-6*	**0.372**	**0.792**	0.040	-0.126	0.057
n-3					
18:3*n-3*	-0.013	0.061	**-0.237**	**-0.701**	-0.141
20:5*n-3*	**-0.849**	-0.151	**0.230**	0.157	-0.049
22:5*n-3*	0.001	0.026	**0.761**	**0.204**	**-0.223**
22:6*n-3*	**-0.475**	-0.076	**0.496**	**0.498**	0.054

Bold indicate strong correlation. EFA: essential fatty acids

Components 1, 3 and 5 from the total plasma differed significantly between the two treatment groups, *P* value of 0.045, $4.8x10^{-6}$ and $2.7x10^{-7}$ respectively. While components 1 and 2 from the erythrocyte phospholipids differed significantly between the two groups with *P* value of $2.8x10^{-7}$ and 0.043 respectively as summarised in **Table 3.11** and **Table 3.12**.

Table 3.11 Comparison of the generated components from plasma fatty acids between the two groups after 3 months intervention.

	Control	Intervention	P^1
	(*n*= 82)	(*n*= 80)	
Component-1	0.14 (-0.05,0.33)	-0.14 (-0.33,0.05)	**0.045**
Component-2	0.02 (-0.20,0.25)	-0.02 (-0.25,0.20)	0.767
Component-3	-0.35 (-0.55,-0.14)	0.36 (0.15,0.56)	**$4.80x10^{-6}$**
Component-4	0.10 (-0.12,0.32)	-0.10 (-0.32,0.12)	0.203
Component-5	0.37 (0.18,0.56)	-0.38 (-0.58,-0.19)	**$2.70x10^{-7}$**
Component-6	0.08 (-0.15,0.30)	-0.08 (-0.30,0.15)	0.342

Univariate analysis of variance was used to perform the comparison.

[1]*P*-value adjusted for age, gender, BMI and ethnicity.

Table 3.12 Comparison of the generated components from erythrocyte phospholipids fatty acids between the two groups after 3 months intervention.

	Control	Intervention	
	(*n*= 82)	(*n*= 80)	*P*[1]
Component-1	0.40 (0.19,0.61)	-0.45 (-0.67,-0.23)	**2.80×10^{-7}**
Component-2	0.17 (-0.07,0.41)	-0.19 (-0.44,0.06)	**0.043**
Component-3	-0.10 (-0.34,0.13)	0.12 (-0.14,0.37)	0.215
Component-4	-0.08 (-0.30,0.14)	0.09 (-0.15,0.33)	0.309
Component-5	-0.01 (-0.25,0.23)	0.01 (-0.24,0.26)	0.922

Univariate analysis of variance was used to perform the comparison.

[1]*P*-value adjusted for age, gender, BMI and ethnicity.

Figure 3.5 shows the ROC curves for the plasma PCA components and **Figure 3.6** for the erythrocyte lipid components. Component 5 for plasma was the most powerful at discriminating between diets whereas component 1 for erythrocytes was most powerful predictor of compliance.

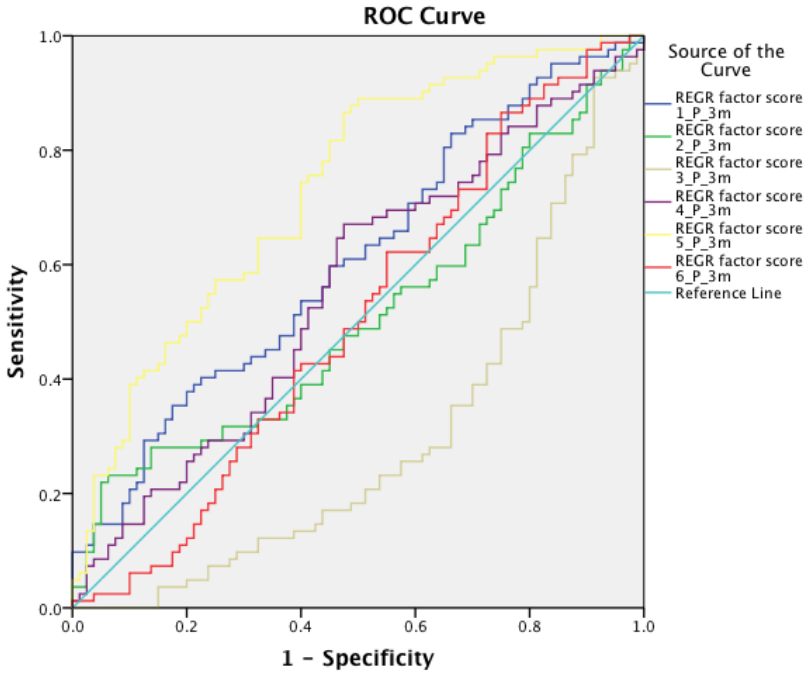


Figure 3.3 Receiver operator characteristics for components from PCA of plasma according to intervention or control diet.

Table 3.13 Area under the curve of the ROC analysis for plasma PCA component.

Area Under the Curve

Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
REGR factor score 1_P_3m	0.607	0.044	0.018	0.521	0.694
REGR factor score 2_P_3m	0.510	0.046	0.828	0.420	0.600
REGR factor score 3_P_3m	0.297	0.041	0.000	0.216	0.378
REGR factor score 4_P_3m	0.557	0.045	0.208	0.468	0.646
REGR factor score 5_P_3m	0.733	0.039	0.000	0.656	0.809
REGR factor score 6_P_3m	0.506	0.046	0.901	0.416	0.596

[a]Under the nonparametric assumption.

[b]Null hypothesis: true area = 0.5.

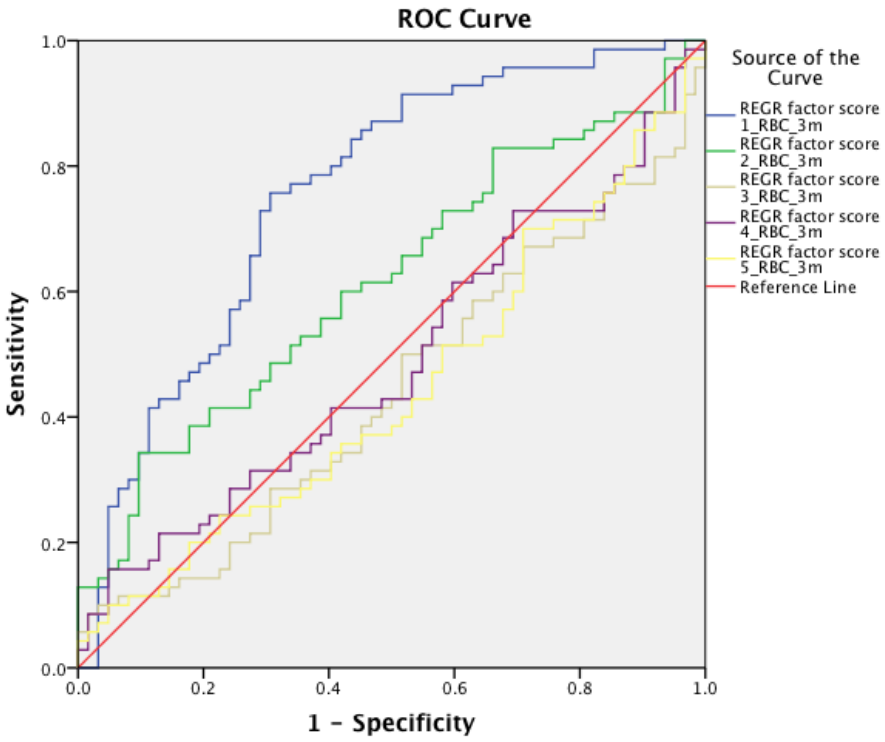


Figure 3.4 Components from PCA of erythrocytes according to intervention or control diet

Table 3.14 Area under the curve of the ROC analysis for RBC PCA component

Area Under the Curve					
Test Result Variable(s)	Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
				Lower Bound	Upper Bound
REGR factor score 1_RBC_3m	0.756	0.043	0.000	0.672	0.840
REGR factor score 2_RBC_3m	0.616	0.049	0.022	0.521	0.712
REGR factor score 3_RBC_3m	0.441	0.050	0.243	0.343	0.539
REGR factor score 4_RBC_3m	0.490	0.051	0.845	0.391	0.589
REGR factor score 5_RBC_3m	0.445	0.050	0.280	0.347	0.544

[a]Under the nonparametric assumption.
[b]Null hypothesis: true area = 0.5.

3.7.7 Relationship of biomarkers to carotid intima-medial thickness (CIMT).

Baseline carotid diameter, intima-media thickness (IMT) and presence of atherosclerotic plaque in participants as detected by high resolution ultrasound stratified by gender are illustrated in **Table 3.15**. Left and right minimal carotid diameters were higher in males than females. The same applies to left and right maximal carotid diameters. Left and right anterior IMT and posterior IMT were also higher in males than females.

Table 3.16 summarizes the CIMT thickness stratified by omega-3 tertiles. There was no significant difference between the three levels of omega-3 index for the posterior or the anterior vessels.

Table 3.15. Carotid diameter, intima-media thickness (IMT) and presence of atherosclerotic plaque in participants as detected by high resolution ultrasound.

	Left		Right	
	Males	Females	Males	Females
	n=59	*n*=91	*n*=59	*n*=91
Minimal carotid diameter (mm)	6.86±0.49	6.32±0.66	7.08±0.60	6.47±0.61
Maximal carotid diameter (mm)	7.33±0.57	6.79±0.68	7.58±0.65	6.94±0.65
Anterior IMT (mm)	0.61±0.10	0.60±0.11	0.63±0.11	0.61±0.10
Posterior IMT (mm)	0.58±0.14	0.56±0.13	0.55±0.11	0.52±0.09
Carotid plaque (%)[1]	10.1%	6.5%	5.1%	3.2%

[1]% of total number.

There were significant associations of age, systolic BP and total cholesterol:HDL-cholesterol on IMT measures.

Table 3.16 CIMT thickness stratified by erythrocytes omega-3 index tertiles.

	Omega-3 index			
IMT	Low	Medium	High	*P*-value for trend
Left anterior	0.61 (0.58,0.63)	0.60 (0.58,0.63)	0.60 (0.57,0.63)	0.875
Left posterior	0.57 (0.54,0.60)	0.56 (0.53,0.59)	0.60 (0.56,0.63)	0.288
Right anterior	0.63 (0.60,0.65)	0.61 (0.58,0.63)	0.62 (0.59,0.65)	0.765
Right posterior	0.53 (0.51,0.56)	0.52 (0.50,0.54)	0.55 (0.52,0.58)	0.309

Values are mean (95% CI).

Analysis adjusted for age, gender, SBP, TC:HDL, carotid diameter.

3.8 Discussion

The aim of the present study was to investigate the influence of adherence to dietary modification approach versus habitual UK diet on CVD risk and to develop an overall biomarker of compliance to dietary fat modification using fatty acid proportions of erythrocyte phospholipids and total plasma as indicators of intake. The distribution of fatty acids in our study was in the range expected based on other published reports for both erythrocytes and plasma. Among all the SFA, palmitic and stearic acids showed weak, but significant correlation, 0.197 and 0.179 respectively. A substantial proportion of palmitic and stearic acids may be converted to oleic acid. The other SFA, myristic (C14:0) and arachidic (C20:0) acids were not detected in the erythrocytes. Dietary contribution of palmitoleic acid is known to be negligible, with the exception of macadamia nut and fish oil, and endogenous synthesis in the liver (delta-9 desaturation of palmitic acid) is responsible for the circulating levels of this fatty acid. Its synthesis is known to be suppressed when PUFA intake is high (Sanders, 1988). However, all PUFAs from the *n*-6 and *n*-3 families showed a significant correlation with the exception of (18:3*n*-3). It appeared that the control diet, which had a greater intake of dairy fat (butter spread, full-fat milk and unrestricted cheese intake), may have resulted in a slightly greater intake of LA. This would explain why component 5, which was also driven by the level of DGLA and AA, could be used to discriminate the intervention from control diets. TFA showed a very strong significant correlation between the two biomarkers. However, levels were very low. The slightly higher values in the control

group, which might be explained by the selective uptake by the erythrocytes, showed higher levels of TFA. TFAs are known to be atherogenic and proportions of TFA in erythrocyte phospholipids might be used as a long term indicator of intake.

After three months following an integrated dietary approach, 16:1*n*-7, 18:1*n*-7, TFA and 18:3*n*-3 in erythrocyte phospholipids and 20:0, 18:1*n*-7, 18:2*n*-6 and 22:4*n*-6 in plasma did not differ between the two study groups. Palmitoleic acid was lower in the plasma of the intervention group.

LA was significantly lower in erythrocytes, but not in the plasma of the intervention group. ALA was significantly lower in the plasma, but showed no difference in erythrocyte phospholipids. EPA and DHA were significantly higher in the two biomarkers, which indicate adherence to the dietary intervention. Levels of EPA and DHA reflect dietary intake of food items rich in these fatty acids such as oily fish in addition to active elongation and desaturation of precursor fatty acids. Using the plasma fatty acid composition to estimate the intake of EPA and DHA based on the dose response relationships in the MARINA study suggested an estimated intake of 0.3 g/d for EPA *vs* an estimated dietary intake 0f 0.4 g/d. However, the mean estimated intake of DHA was 0.2 g *vs* a measured intake of 0.8 g from the food diaries. It is possible that the slightly higher intake of dairy fat and meat products and the slightly lower intake of LA on the control diet resulting in smaller differences in DHA in plasma. High levels of DHA have been found in animals fed a mixture of butter and lard compared with diet high in polyunsaturated margarine (Sanders *et al*. 1984).

Performance of PCA on fatty acid proportions after three months on the diets enabled the recognition of components in the plasma and erythrocyte lipids that discriminated

between diets. These components provide a more concise indicator of change in fatty acid profile instead of changes in individual fatty acids. In plasma component 5 was most informative and component 1 for erythrocytes. The proportion of total SFA in plasma was also able to discriminate between the control and intervention diet but the proportion of SFA in erythrocytes lacked that capacity.

3.9 Conclusion

This dietary intervention study was successful in achieving its dietary targets. Changes in EPA seemed to be more sensitive to diet than DHA. The proportion of total SFA did enable discrimination between the control and intervention group probably because it is sensitive to reductions in PUFA intake. The results of the fatty acid analysis indicated that PCA analysis could be useful in identifying individuals who have made changes in their dietary fat intake that are consistent with advice to modify fat intake.

Chapter 4: Heritability of fatty acid composition of adipose tissue and plasma

4.1 Introduction

It is is uncertain to what extent genetic factors influence the fatty acid composition of lipids. Twin studies can be used to investigate the genetic and environmental influences on traits and diseases in the population. The genotype is determined by differences in the sequences of DNA whereas the phenotype is what is expressed. The phenotype expressed is dependent on the genotype and is also influenced by epigenetic effects which appear to have their greatest effects in early growth and development. Twin studies utilize the unique degree of shared genetic and environmental factors among the two kinds of twin pairs, monozygotic (MZ) and dizygotic (DZ) twins. MZ or identical twins are characterised by sharing common set of genes, while DZ or non-identical twins share 50% of their genes on average. Furthermore, both types of twins share the same uterine environment, age and features of early and late environment. These characteristics enable an estimation of the variation of traits into genetic, shared environmental and random environmental components. Estimation of the amount of the contribution of individual component of variation can be achieved by using quantitative analytical techniques to twin data. This gives an estimate of heritability, which has been defined as "a measure of the extent to which phenotypic variation in the population can be explained by genetic variation" (MacGregor *et al*. 2000).

Twin studies have been utilized in association and linkage studies, gene expression studies and gene-environment interactions. As far as could be ascertained no previous studies in twins have investigated the hereditability of the fatty acid composition of

adipose tissue and plasma. Two studies were found where heritability of fatty acids was investigated. One study explored the effect of heritability on proportions of branched chain fatty acids in co-twin study design, which estimated heritability by comparing the associations of traits within dizygotic and monozygotic twins (Stewart *et al*. 1986). Heritability of metabolites was also investigated (Shah *et al*. 2009). This chapter presents evidence for a significant independent effect of heritability on adipose tissue and plasma fatty acid composition in a subset of 570 healthy female twins of European descent aged 25-80 years from the TwinsUK cohort.

4.2 Purpose of the study

The aim of this study was to determine the influence of environmental (dietary intake) and additive genetic factors (heritability) on adipose and plasma lipids biomarkers of fatty acid intake using a co-twin design. These aims were achieved by the following objecives:

1. To conduct detailed measurements of fatty acid composition of samples of adipose tissue and plasma from pairs of monozygotic and dizygotic twins recruited to the TwinsUK cohort.
2. To calculate heritability of plasma and adipose tissue fatty acid composition.
3. To assess the relationship between dietary estimates of fatty acid intake from FFQ with these biomarkers.
4. To estimate dietary intake of long chain PUFA (LC-PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from the plasma fatty acid composition based on calibration from a long-term dose response study (MARINA).
5. To investigate the influence of variants in candidate SNPs for the for *FADS1*, fatty acid desaturase, and *SCD-1*, stearoyl-CoA desaturase genes.

Hypothesis (1)

Additive genetic factors (A) have a significant influence on fatty acid composition of adipose tissue and plasma of twins.

Hypothesis (2)

SNPs at *SCD-1* gene and *FADS1* gene cluster influence plasma and adipose tissue proportions of MUFA and PUFA respectively.

4.3 Study participants and design

The present study was a cross-sectional observational study and took advantage of the fact that a subgroup of female twin participants were invited to provide samples of adipose tissue for gene expression studies. During the collection of biopsies, samples were set aside specifically for fatty acid analysis. All participants were twins enlisted in the TwinsUK registry, which is a national register of adult twins. The recruitment methodology has been described in detail by Spector and Williams. (2006). Twins were recruited to the registry as volunteers through a series of media campaigns and were not chosen for disease-specific investigations. The participant attended St Thomas' Hospital for initial clinical assessment between 1993 and 2004. Food frequency questionnaires were completed between 1995 and 2007 with most (84%) being completed in 2007 with the remainder being completed between 1995 and 2001. There were no differences in % energy from total fat between years but SFA and TFA intake were marginally lower (0.48% and 0.06% energy respectively) and PUFA higher (0.39% energy) in FFQs completed in 2007. In this study, adipose tissue biopsies were obtained in 2008 from MZ (*n*= 230) and DZ (*n*= 340) pairs of twins with majority being postmenopausal women (UK residents) without metabolic disorders that were likely to alter the dietary intake or require its modification.

4.4 Methods

4.4.1 Analytical methods

Adipose tissue was collected from participants by biopsies where a small incision was made under local anaesthesia and samples of up to 0.5g were obtained from the abdominal subcutaneous tissue. Approximately, 50mg were set aside for lipid analysis, wrapped in aluminium foil and stored at -80˚C until analysed. Fasting blood samples were collected in an EDTA containing tubes and centrifuged and the plasma sample was separated and stored at -80˚C. Up to 5mg adipose tissue was incubated with 2ml mixture of 1.25mol HCL/L methanol: toluene (4:1 by volume) in a glass culture tube fitted with a Teflon lined screw cap and incubated at 60˚C for 2hrs. After which 5ml of 60g potassium carbonate/L water was added, the sample vortex mixed and centrifuged at 1500rpm for 10min to enable clear phase separation. Upper toluene phase was carefully collected using a glass pipette or a Hamilton syringe and transferred into an amber glass GLC vial and diluted with 500µl toluene. Fatty acid methyl esters (FAME) were separated on an Agilent 7890 Gas Chromatograph (Agilent Technologies) fitted with a flame ionisation detector with a 60m BPX70 SGE fused silica column and 0.25mm diameter (SGE, Cat no. 054623 Milton Keynes, UK) in split mode 50:1 with hydrogen as carrier gas (1ml/min). Injector and oven temperatures were at 250°C and at 180°C respectively. FAME standards were obtained from Sigma Aldrich (Cat no.1891-1, 189-2 and 189-3) and secondary reference standards of methyl esters of fish oil concentrate (MaxEPA, Seven Seas Hull, UK) were used to identify the fatty acids. The

identity of fatty acids for which standards were not available was estimated using plots of the retention times as described by Christie. W (1990) to obtain the equivalent chain lengths and *n*-9/*n*-6/*n*-3 ratios. Quality control was maintained by running a matrix standard of sample of adipose tissue FAME with each run. Chromatograms were evaluated using ChemStation software (version 8.04) and the output exports via visual basic application into excel for data analysis.

Plasma total fatty acids were methylated using a modification method of Lepage & Roy as described by Sanders *et al*. (2006a), substituting toluene for benzene and using pentadecanoic acid (C15:0) as an internal standard. FAME were separated on a BPX70 column 25m x 220µm film thickness x0.25µm internal diameter (SGE, Cat no. 054602 Milton Keynes, UK) on an Agilent 7890 (Agilent Technologies, Cheshire, United Kingdom). Chromatograms were integrated using ChemStation software and exported into Microsoft Excel.

4.4.2 Dietary assessment

Participants completed 131-items FFQ described elsewhere (Teucher *et al*. 2007). Briefly, the FFQ was used earlier in the EPIC study and an established database of nutrients composition used to derive dietary intake. Nutrient intakes were calculated based on data from the 5th Edition of McCance & Widdowson's The Composition of Foods (Holland *et al*. 1991) and the seventh supplement (MAFF, 1998). Participants were excluded if answers for more than 10 food items were left blank and if the ratio of total energy intake to estimated basal metabolic rate (using Harris-Benedict equation)

exceeds 2 standard deviations from the mean of that ratio (<0.52 or >2.58) (Teucher *et al*. 2007). Nutrient intake data were available from the TwinsUK Registry at St Thomas' Hospital.

4.4.3 Zygosity, body composition, biochemical analyses and SNPs genotyping

Information on zygosity was determined by standardized questionnaire and confirmed by DNA fingerprinting. Height was measured to the closest 0.5cm using a wall-mounted stadiometer. Weight (only light clothing allowed) was measured to the closest 0.1 kg using digital scales. Fasting blood samples were collected from subjects after a minimum 8hrs overnight fast for determination of fasting blood lipids, glucose and insulin. Serum samples were stored at −45°C until analyzed using a CobasFara machine (RocheDiagnostics, Lewes, UK). A colorimetric enzymatic method was used to determine total cholesterol (TC), triacylglycerols (TAG), and high density lipoprotein (HDL-C) cholesterol levels. The latter was measured after precipitation from chylomicron, low density lipoprotein (LDL), and very low-density lipoprotein (VLDL) particles by magnesium and dextransulphate and LDL cholesterol was estimated using the Friedewald equation (total cholesterol – HDL cholesterol-TAG/2.2). Insulin was measured by immunoassay (Abbott Laboratories, Maidenhead, UK) and glucose was measured on an Ektachem 700 multichannel analyzer using an enzymatic colorimetric slide assay (Johnson and Johnson ClinicalDiagnostic Systems, Amersham, UK) in an accredited clinical laboratory. HOMA2-IR was calculated using Homeostasis Model

Assessment (HOMA), a software implementation of the HOMA2 model developed by Diabetes Trials Unit, University of Oxford (http://www.dtu.ox.ac.uk/homacalculator/)

Genotypes were extracted from whole genome analysis. These were provided by the St Thomas' TwinsUK study group. SNPs were genotyped using Illumina 610k and Illumina 317K arrays as described elsewhere (Illig *et al*. 2010).

4.5 Statistical analysis

Standard distributional checks were made using Q-Q plots in SPSS and where the data did not follow a normal distribution a natural logarithmic transformation was attempted and the distribution checked. Statistical analysis was performed using SPSS version 20.0 for Windows (SPSS Inc, Chicago, IL, USA). Spearman's correlation was used to assess the associations. Data reduction conducted using principal component analysis (PCA). Obtained components were then subjected to varimax rotation to calculate orthogonal solutions.

In order to estimate intakes of EPA and DHA in the samples, data was used from a dose response study (MARINA study, *see* Chapter 5). In that study participants had been randomly allocated different doses of EPA+DHA and measurements of EPA and DHA in plasma following supplementation had been made. The data were fitted using a linear regression equation and the slope and intercept estimated using PRISM (GraphPad version 5).

Linear structural equation modelling was used to estimate the genetic and environmental components of variance which decays variations in a given phenotype into additive genetic effects (A), environmental effects shared in common (C) and non-shared environmental effects (E) using the Mx software package (Neale *et al*. 2002). The goodness-of-fit analysis was completed by calculating the difference between the model fitted and the fully saturated model (Mohammed *et al*. 2005; Teucher *et al*. 2007). Adjustment allowing for differences in age and BMI were done using the linear regression analysis module within SPSS and save the residuals as a new variable. The distribution of the residual was then checked for normality and where appropriate was log-normalised before further analysis. **Figure 4.1** illustrates the mathematical concept used to estimate heritability by ACE model

All genotype distributions were tested for deviation from the Hardy-Weinberg equilibrium by a χ^2 test with 1 df ($P > 0.05$). Owing to the relatively small sample size, SNP genotype association analyses were based on a dominant model. Data are presented as means or geometric means ± SD adjusted for confounding effect of age, BMI and ethnicity. Multivariate analysis of variance was used to examine the genotype associations with proportions of fatty acids and plasma lipids to allow adjustment for multiple testing, while univariate analysis of variance was used to test genotype associations with surrogate measures of indices of desaturases activities. The adjustment for within pair effects was accomplished by DTR team using Merlin (Multiple Engine

for Rapid Likelihood Inference) software which was download from the website www.genomeutwin.org/member/cores.stat/linkage/merlin.html [accessed 4/7/2013].

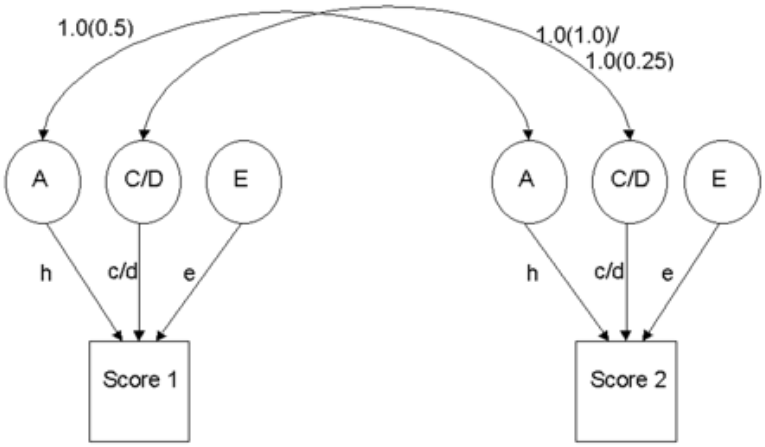


Figure 4.1 ACE path modelling. Path model for univariate analysis of a twin study. The observed phenotypes of twin 1 and twin 2 (score 1 and score 2) are represented in *squares.* Latent factors are represented in *circles*: A, C, D, and E are the additive genetic, dominant genetic, and common and individual environmental influences common to Score 1 and Score 2. A and D are correlated by a factor of 1.0 for MZ twins and 0.5 and 0.25, respectively, for DZ twins; C is correlated by a factor of 1.0 for both MZ and DZ twins (the equal environment assumption). Regression coefficients of the observed variables on the different latent factors are also shown in *lowercase*: for example, h is the regression coefficient of the additive genetic effect. C and D cannot be estimated simultaneously. From (Hammond *et al*. 2001)

4.6 Results

4.6.1 Characteristics of the study participants

The characteristics of the female study participants are shown in **Table 4.1**. The estimated intakes of SFA, MUFA, PUFA and TFA from the FFQ were 11.5%, 10.5%, 7.2% and 0.9% dietary energy respectively and comparable to UK women reported elsewhere using the same methodology in the Oxford and Norfolk cohorts of the EPIC study (Davey *et al*. 2003; Bingham *et al*. 2001). Plasma total and LDL-C were moderately elevated and similar to values reported in nationwide surveys for women of comparable age (Henderson *et al*. 2003).

Table 4.1 Details of the female twins participating in the study.

	Monozygotic (*n*= 230)	Dizygotic (*n*= 340)	*P*-value[1]
Age	60.2 ± 8.2	57.5 ± 9.4	<0.001
Height (cm)	160.9 ± 6.2	161.8 ± 5.7	0.174
Weight (kg)	68.1 ± 14.7	69.3 ± 14.2	0.232
BMI (kg/m2)	26.3 ± 5.1	26.4 ± 5.0	0.975
Birth weight	2.2 ± 0.5	2.4 ± 0.6	0.001
Omega-3 supplement use	44 (19.1%)	66 (19.4%)	0.512
Energy (MJ/d)	8.32 ± 2.62	8.09 ± 2.37	0.355
Protein (% energy)	17.3 ± 2.3	17.3 ± 2.4	0.994
Carbohydrates (% energy)	50.8 ± 5.4	50.5 ± 6.3	0.790
Fat (% energy)	31.8 ± 4.8	31.7 ± 5.1	0.736
SFA (% energy)	11.8 ± 2.6	11.4 ± 2.8	0.141
MUFA (% energy)	10.5 ± 1.8	10.5 ± 2.0	0.648
PUFA (% energy)	7.1 ± 1.7	7.3 ± 1.8	0.107
TFA (% energy)	0.9 ± 0.3	0.8 ± 0.4	0.137
TC (mmol/L)	5.61 ± 1.08	5.61 ± 1.04	0.474
LDL-C (mmol/L)	3.21 ± 1.01	3.29 ± 0.96	0.160
HDL-C (mmol/L)	1.86 ± 0.47	1.83 ± 0.43	0.615
TC:HDL-C	3.20 ± 1.08	3.20 ± 0.85	0.723
Non HDL-C (mmol/L)	3.74 ± 1.12	3.78 ± 1.04	0.365
TAG (mmol/L)[2]	1.16 ± 0.59	1.08 ± 0.51	0.186
Insulin (pmol/L)[2]	55 ± 44	49 ± 68	0.202
Glucose (mmol/L)[2]	5.1 ± 0.6	4.9 ± 0.7	0.042
HOMA2 IR	1.03 ± 0.80	0.91 ± 1.09	0.100

Values are mean ± SD or number of subjects (%).

[1] Probabilities are from analysis of covariance adjusted for age, BMI, ethnicity or Chi-squared test for frequencies.

[2]Non fasting values from 9 participants were excluded.

MZ: monozygotic twins; DZ: dizygotic twins; BMI: body mass index; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: trans-fatty acids; TC: total cholesterol; LDL-C: LDL cholesterol; HDL-C: HDL cholesterol; TC:HDL-C: ratio of total to HDL cholesterol; Non HDL-C: non HDL cholesterol; TAG: triacylglycerol; HOMA2: The Homeostasis Model Assessment calculated using a software implementation of the HOMA2 model developed by Diabetes.

4.6.2 Fatty acid composition of adipose tissue: A comparison with published reports

Figure 4.2 shows a typical GLC trace of the adipose tissue. The analyses illustrated clear separation of *cis* and *trans* isomers of 18:1 and a significant number of minor components. The mean values with SD are shown in **Table 4.2** and for comparison data from two earlier studies in UK subjects (Craig *et al*. 2009) which did not specify the site of biopsy and Bolton-Smith *et al*. (1997) who used upper arm biopsies. The proportion of palmitic acid (16:0) and other SFA (14:0, 18:0) and palmitoleic acid (16:1*n*-7) in the TwinsUK cohort were similar to that in women previously by Bolton-Smith *et al*. (1997). The proportion of LA was greater in the TwinsUK cohort than the women studied by Bolton-Smith but was comparable to the more recent data of Craig *et al*. (2009) probably reflecting the generally higher dietary LA (18:2*n*-6) intake in 2009 *vs*. 1997. This finding would fit in with the observation of a slightly lower proportion of oleic acid in the more recent data. Palmitoleic acid (16:1*n*-7) was present in similar proportions to that reported by Craig *et al*. (2009) but was about one third lower than that reported by Bolton-Smith *et al*. (1997).

A GLC trace of plasma fatty acids is shown in **Figure 4.3**. Good separation of the major fatty acids was obtained. **Table 4.3** summarizes the mean values with their SD and comparisons with women recruited into the MARINA study measured in the same laboratory using an identical method. Slightly higher proportions of SFA and TFA and

lower PUFA were found in the plasma of the TwinsUK compared to participants in MARINA trial (all $P<0.001$; unpaired *t*-test).

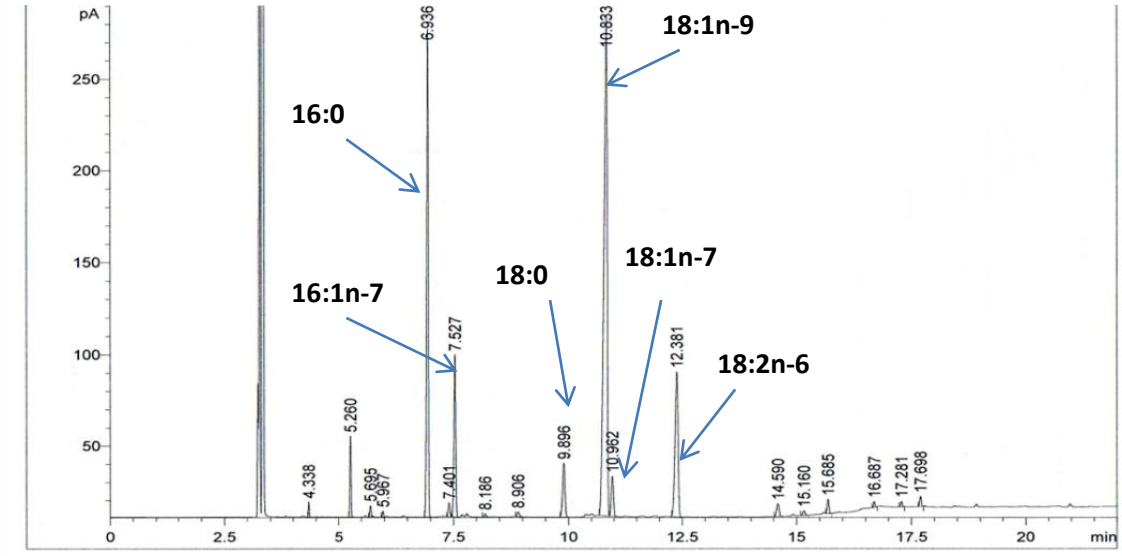


Figure 4.2 A typical chromatogram of the fatty acids methyl esters from adipose tissue. The detector response is shown in picoamps against time in minutes and the numbers on the x-axis indicate the retention time post-injection.

Table 4.2 Comparison of fatty acid proportions (wt%) in adipose tissue with other reports from the United Kingdom.

Fatty acid	Twins F (*n*= 570)	Craig *et al.* 2009 M (*n*= 9), F (*n*= 24)		Bolton-Smith *et al.* 1997 M(*n*= 2308), F (*n*= 2049)[1]
	Mean ± SD	Median	IQR	Mean ± SD
10:0	0.32 ± 0.36	-	-	-
12:0	0.61 ± 0.73	0.5	0.4, 0.6	-
14:0	2.02 ± 0.88	2.8	2.3, 3.2	2.24 ± 0.76
16:0	19.24 ± 2.06	22.2	20.6, 22.9	19.43 ± 2.4
18:0	3.21 ± 0.95	3.7	2.6, 4.5	3.48 ± 1.20
20:0	0.35 ± 0.16	-	-	-
ΣSFA	25.76 ± 3.03	29.4	27.2, 31.4	-
16:1n-7	5.76 ± 1.70	6.4	5.2, 7.6	9.40 ± 2.02
18:1n-9	46.54 ± 3.05	50.4	47.3, 51.8	51.40 ± 2.92
18:1n-7	0.78 ± 0.93	-	-	-
ΣMUFA	53.08 ± 3.44	56.7	54.2, 59.3	-
18:1 trans	0.46 ± 0.33	-	-	-
ΣTFA	0.46 ± 0.33	-	-	-
18:2n-6	12.03 ± 2.42	11.9	10.8, 13.0	9.35 ± 2.50
18:3n-6	-	0.02	0.01, 0.03	0.18 ± 0.09
20:3n-6	0.32 ± 0.18	0.17	0.10, 0.21	0.15 ± 0.06
20:4n-6	0.56 ± 0.19	0.30	0.23, 0.39	0.61 ± 0.15
22:4n-6	0.25 ± 0.17	-	-	-
Σn-6	13.16 ± 2.49	12.4	11.2, 13.6	-
18:3n-3	0.84 ± 0.24	1.1	1.0, 1.3	-
20:5n-3	0.21 ± 0.16	0.02	0.02, 0.07	-
22:5n-3	0.38 ± 0.18	-	-	-
22:6n-3	0.36 ± 0.22	0.09	0.05, 0.20	0.48 ± 0.164*
Σn-3	1.79 ± 0.53	1.4	1.1, 1.7	-
ΣPUFA	14.95 ± 2.71	13.9	12.9, 14.8	-
n-6:n-3	7.75 ± 2.32	0.10		

The data reported are the sum of 22:5n-6 + 22:6*n*-3.

Table 4.3 Comparison of total plasma fatty acids in the female twins compared with female participants recruited into the MARINA study (*see* Chapter 5).

Fatty acid	Twins (*n*= 208)	MARINA (*n*= 210)
10:0	-	-
12:0	-	-
14:0	0.82 ± 0.24	0.75±0.25
16:0	20.94 ± 1.35	20.06±1.29
18:0	7.08 ± 0.58	6.53±0.53
20:0	0.22 ± 0.05	0.24±0.06
ΣSFA	29.07 ± 1.62	27.58±1.36
16:1n-7	2.50 ± 0.73	2.31±0.92
18:1n-9	19.49 ± 2.05	20.43±2.46
18:1n-7	1.54 ± 0.23	1.61±0.26
ΣMUFA	23.53 ± 2.54	24.34±3.20
18:1 *trans*	0.13 ± 0.06	0.07±0.06
ΣTFA	0.13 ± 0.06	0.07±0.06
18:2n-6	26.76 ± 3.65	27.75±4.00
18:3n-6	0.46 ± 0.16	0.46±0.17
20:3n-6	1.60 ± 0.37	1.60±0.35
20:4n-6	6.37 ± 1.32	6.91±1.55
22:4n-6	0.53 ± 0.15	0.58±0.15
22:5n-6	1.06 ± 0.22	1.04±0.31
Σn-6	36.78 ± 3.54	38.34±3.95
18:3n-3	0.62 ± 0.16	0.65±0.20
20:5n-3	1.49 ± 0.83	1.17±0.65
22:5n-3	0.61 ± 0.12	0.64±0.13
22:6n-3	2.48 ± 0.87	2.53±0.69
Σn-3	5.21 ± 1.69	4.99±1.34
ΣPUFA	42.00 ± 3.64	43.32±3.96
n-6:n-3	7.77 ± 2.50	8.20±2.28

Mean values ± SD in wt %.

Analyses determined in the same laboratory at KCL using identical methods.

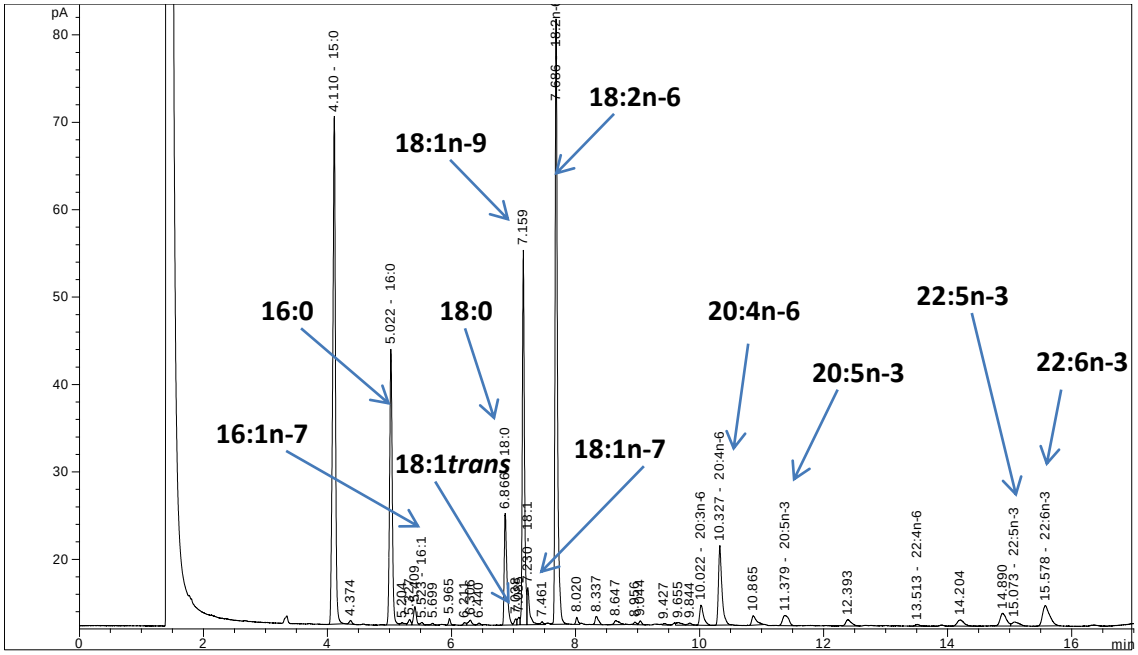


Figure 4.3 Typical chromatogram of fatty acid methyl esters from total plasma lipids The detector response is shown in picoamps against time in minutes and the numbers indicate the reterntion time post-injection.

4.6.3 Estimation of EPA and DHA intake

Using known data on EPA and DHA intake and proportions in their plasma from the female participants from the MARINA study (*see* Chapter 5), intakes of EPA and DHA were estimated in the female twins. The proportion of EPA and DHA were plotted against their levels in diet using a linear regression curve fitting equation in PRISM GraphPad version 5.04 (GraphPad.Com; California). Values of the proportions of fatty acids in plasma lipids were used to interpolate intakes of EPA and DHA in the female twin participants.

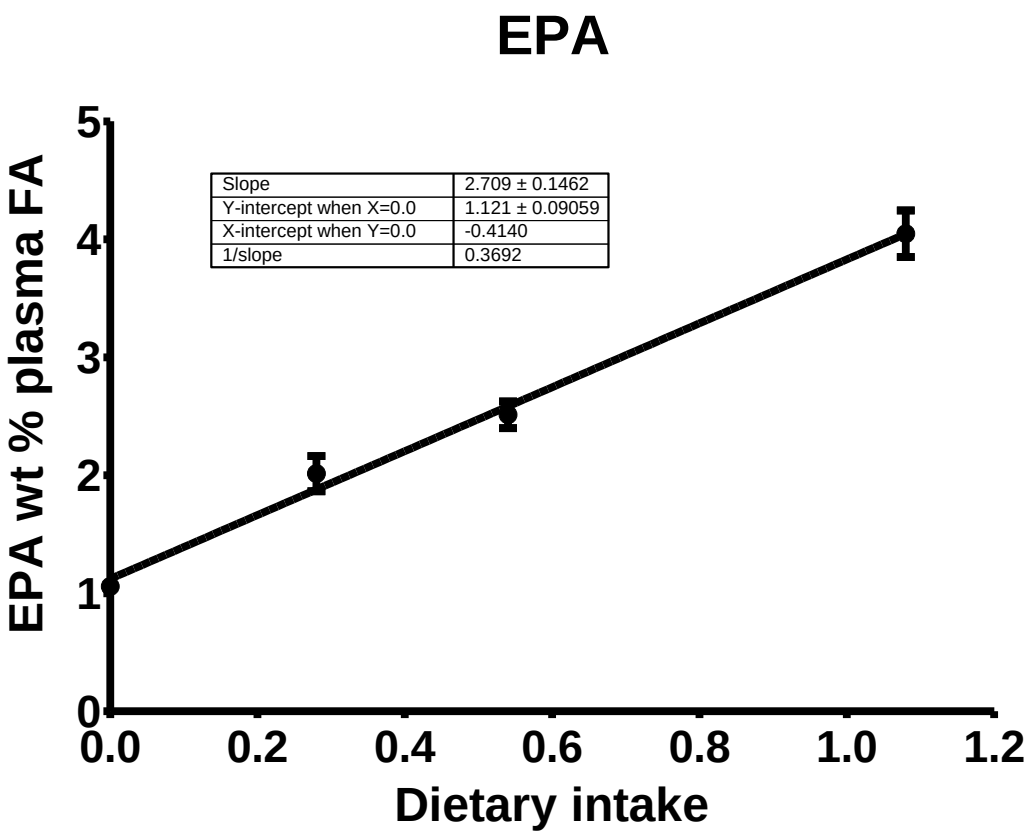


Figure 4.4 Equation developed from MARINA female only samples used to estimate EPA intake.

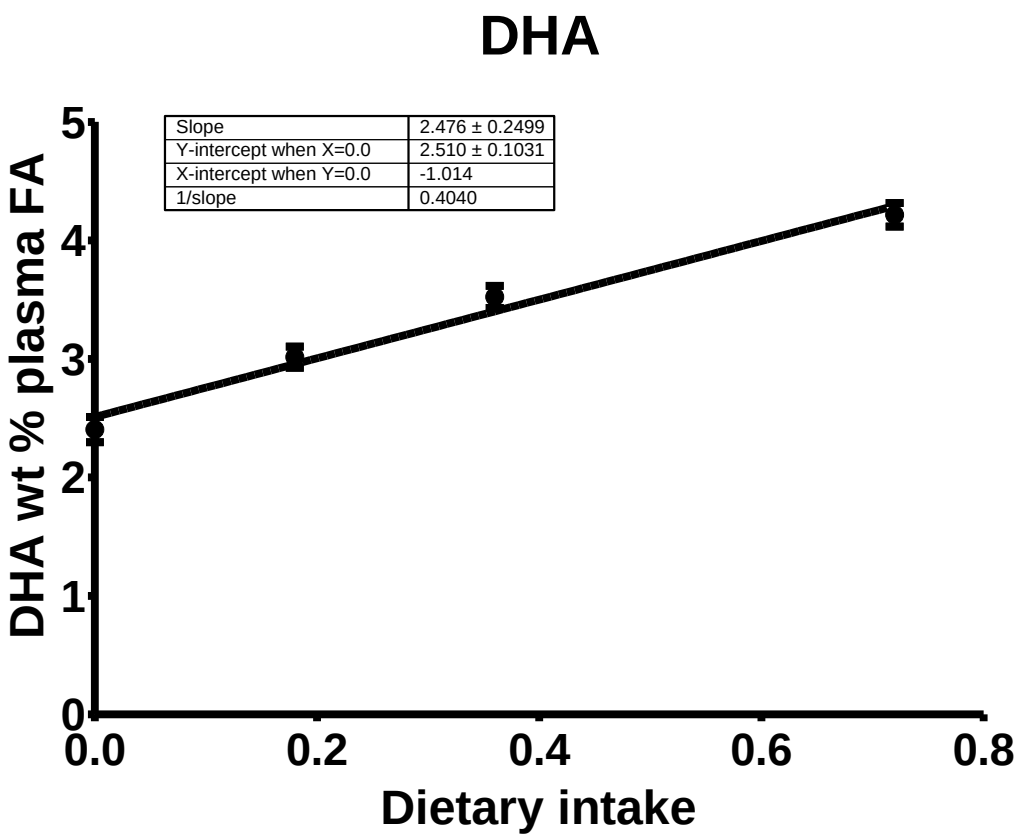


Figure 4.5 Equation developed from MARINA female only samples used to estimate DHA intake.

Estimated intakes of EPA and DHA are shown in **Figure 4.4** and **Figure 4.5** respectively. The mean ± SD intakes of EPA and DHA in female twin participants were 176 ± 28 mg/d and 131 ± 23 mg/d respectively. Thus, total intake of EPA + DHA in this cohort of female twin was estimated to be 307 ± 48 mg/d. Stratification by zygosity did not show any significant difference between the two groups. A comparison of the levels in participants who reported taking dietary supplements was made (**Table 4.4**). The estimated intake of DHA was greater (P<0.05) in those reporting taking supplement than in those who did not. The difference in estimated intakes of EPA did not differ. Cod-liver oil is the most widely used omega-3 supplement and this contains more DHA than EPA. A typical one a day cod-liver oil capsule contains about 0.2-0.3 g of EPA+DHA. Furthermore, EPA levels are more readily influenced by the intake of ALA than DHA levels.

Table 4.4 Estimates of intake of EPA and DHA based on plasma concentrations.

	Omega-3 supplement use			
	Non-users (*n*= 171)		Supplement users (*n*= 37)	
	Mean	SD	Mean	SD
20:5n-3	163	239	235	401
22:6n-3	116	205	201	334*

**P*=0.043 significant difference between supplement users and non-users by two sample *t*-test.

4.6.4 Fatty acid proportions in adipose tissue and plasma

The fatty acid composition of the adipose tissue of the study participants stratified by zygosity is summarized in **Table 4.5**. There were no significant differences between MZ and DZ twins in fatty acid proportions. Oleic acid (18:1*n*-9) was the dominant fatty acid accounting for 46.5% of the total, palmitic acid (16:0) accounted for 19.2% of the fatty acids and only small amounts (3.2%) of stearic acid (18:0) were present. Only traces of *trans* isomeric fatty acids (mainly 18:1 *trans* isomers) were found in the samples. Palmitoleic acid (16:1*n*-7) presumably derived by desaturation of palmitic acid in adipose tissue accounted for 5.7% of the total fatty acids. Linoleic acid (18:2*n*-6, LA) and α-linolenic acid (18:3*n*-3, ALA) accounted for 12.1% and 0.85% of the fatty acids respectively. Small amounts of their more unsaturated derivatives notably gammalinolenic acid (18:3*n*-6; GLA, 0.46%), dihomogammalinolenic (20:3*n*-6, DGLA; 0.28%), arachidonic (20:4*n*-6; AA; 0.56%), adrenic (22:4*n*-6; 0.22%) acids in the *n*-6 series and eicosapentaenoic (20:5*n*-3; EPA;0.18%), docosapentaenoic (22:5*n*-3, DPA ; 0.35%) and docosahexaenoic (22:6*n*-3, DHA; 0.31%) acids in the *n*-3 series were present.

The present data set indicates minimal amounts of *trans* fatty acids and provides detailed information on the proportion of LC *n-6* and *n-3* PUFA in adipose tissue.

Table 4.6 shows the fatty acid proportions of the plasma. There were no significant differences between MZ and DZ. PUFA account for about 42% of the total fatty acids and were the most abundant species represented mainly by LA, which constituted

26.75% of all fatty acids followed by AA and DHA, 6.36% and 2.49% respectively. The rest of the PUFA ranged from 1.60% for DGLA, 1.06% for osbond acid (22:5*n*-6), 1.32% for EPA, 0.62% for ALA, 0.61% for DPA, 0.51% for adrenic acid and 0.46% for GLA. This was followed by SFA, which constituted 29.02% and palmitic acid was the most abundant fatty acid with 20.93% of all SFA. Followed by stearic (7.08%), myristic (0.82%) and trace amounts of arachidic (0.22%) acids. MUFA accounted for 23.43% with highest proportions of oleic acid (19.50%) proceeded by palmitoleic (2.50%) and vaccinic (1.54%) acids. Only trace amounts of TFA were detected and these were mainly 18:1 isomers (0.13%). Other minor components included branched chain fatty acids (C15:0 and C17:0) and small amounts of conjugated linoleic acid.

Table 4.5 Fatty acid composition of adipose tissue of female twins

Fatty acid	All subjects[1]	Monozygotic[2]	Dizygotic[3]
SFA	25.75 ± 3.02	25.82 ± 3.02	25.71 ± 3.03
10:0[§]	0.19 ± 0.36	0.21 ± 0.30	0.17 ± 0.40
12:0[§]	0.41 ± 0.73	0.42 ± 0.75	0.40 ± 0.73
14:0	2.03 ± 0.87	2.02 ± 0.89	2.03 ± 0.85
16:0	19.24 ± 2.06	19.26 ± 2.02	19.23 ± 2.09
18:0	3.21 ± 0.95	3.21 ± 1.00	3.22 ± 0.91
20:0	0.36 ± 0.16	0.36 ± 0.15	0.35 ± 0.16
MUFA	53.09 ± 3.44	52.99 ± 3.52	53.16 ± 3.39
16:1*n*-7[§]	5.79 ± 1.66	5.71 ± 1.73	5.84 ± 1.62
18:1*n*-9	46.54 ± 3.05	46.47 ± 2.97	46.60 ± 3.10
18:1*n*-7	0.43 ± 0.94	0.43 ± 0.97	0.43 ± 0.92
TFA			
18:1 *trans* isomers[§]	0.45 ± 0.30	0.45 ± 0.33	0.45 ± 0.27
PUFA	14.95 ± 2.71	14.75 ± 2.76	15.08 ± 2.67
18:2*n*-6	12.05 ± 2.37	11.92 ± 2.31	12.14 ± 2.40
20:3*n*-6[§]	0.28 ± 0.18	0.29 ± 0.17	0.28 ± 0.18
20:4*n*-6	0.32 ± 0.18	0.56 ± 0.18	0.57 ± 0.19
22:4*n*-6[§]	0.22 ± 0.17	0.21 ± 0.18	0.22 ± 0.17
18:3*n*-3	0.85 ± 0.23	0.83 ± 0.21	0.86 ± 0.23
20:5*n*-3[§]	0.18 ± 0.15	0.17 ± 0.16	0.18 ± 0.15
22:5*n*-3[§]	0.35 ± 0.18	0.35 ± 0.18	0.35 ± 0.18
22:6*n*-3[§]	0.31 ± 0.22	0.30 ± 0.25	0.31 ± 0.20

[1] *n*= 570, [2]*n*= 230, [3]*n*= 340

Values are mean ± SD or GM ± SD (for logged variables) wt % total fatty acids.
Values were log-transformed for 10:0, 12:0, 18:1trans, 18:1*n*-7, 20:3*n*-6, 22:4*n*-6, 20:5*n*-3, 22:5*n*-3, 22:6*n*-3.
[§] Indicates data were transformed using natural logarithms.

No significant difference between monozygotic and dizygotic twins from analysis of variance adjusted for age, gender, BMI and ethnicity.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-fatty acids.

Table 4.6 Fatty acid composition of plasma of female twins.

Fatty acid	All subjects[1]	Monozygotic[2]	Dizygotic[3]
SFA[§]	29.02 ± 2.58	28.76 ± 3.55	29.00 ± 1.57
14:0	0.82 ± 0.24	0.80 ± 0.23	0.84 ± 0.24
16:0	20.93 ± 1.35	20.93 ± 1.38	20.94 ± 1.34
18:0	7.08 ± 0.58	7.14 ± 0.57	7.04 ± 0.59
20:0[§]	0.22 ± 0.05	0.23 ± 0.04	0.22 ± 0.06
MUFA	23.43 ± 3.01	23.23 ± 3.60	23.57 ± 2.52
16:1*n*-7	2.50 ± 0.73	2.40 ± 0.68	2.57 ± 0.76
18:1*n*-9	19.50 ± 2.05	19.55 ± 2.11	19.46 ± 2.02
18:1*n*-7	1.54 ± 0.23	1.55 ± 0.24	1.54 ± 0.23
TFA			
18:1 *trans* isomers	0.13 ± 0.06	0.13 ± 0.07	0.13 ± 0.06
PUFA	41.79 ± 4.66	41.54 ± 5.86	41.96 ± 3.58
18:2*n*-6	26.75 ± 3.66	26.88 ± 3.47	26.66 ± 3.80
18:3*n*-6	0.46 ± 0.16	0.44 ± 0.15	0.48 ± 0.16
20:3*n*-6	1.60 ± 0.37	1.59 ± 0.40	1.61 ± 0.35
20:4*n*-6	6.36 ± 1.32	6.32 ± 1.27	6.39 ± 1.36
22:4*n*-6[§]	0.51 ± 0.15	0.49 ± 0.11	0.53 ± 0.17
22:5*n*-6	1.06 ± 0.22	1.09 ± 0.24	1.04 ± 0.20
18:3*n*-3	0.62 ± 0.16	0.63 ± 0.18	0.62 ± 0.14
20:5*n*-3[§]	1.32 ± 0.83	1.30 ± 0.86	1.33 ± 0.81
22:5*n*-3	0.61 ± 0.12	0.60 ± 0.11	0.62 ± 0.12
22:6*n*-3	2.49 ± 0.87	2.49 ± 0.81	2.48 ± 0.91

[1] *n*= 208, [2] *n*= 86, [3] *n*= 122

Mean ± SD or GM ± SD (for logged variables)in wt % total fatty acids.
Values for 20:0, 18:1trans, 22:4*n*-6, 20:5*n*-3, SFA were log transformed prior to analysis.
[§] Indicates data were transformed using natural logarithms.

No significant difference between monozygotic and dizygotic twins from analysis of variance adjusted for age, gender, BMI and ethnicity.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: *trans*-fatty acids.

4.6.5 Correlations between fatty acids proportions in adipose tissue and plasma and those in diet

Table 4.7 shows the correlations between dietary intake and proportions of different fatty acid classes in adipose tissue. No relationships were seen with SFA and MUFA intakes expressed as percentage of the dietary energy intake but significant positive relationships were seen with TFA (ρ= 0.174, P= 1.85×10^{-4}) and PUFA (ρ= 0.287, P= 1.67×10^{-11}) and an even stronger relationship was seen for the ratio of PUFA to SFA (P:S ratio; ρ= 0.331, P= 5.5×10^{-15}) in diet with that of the proportions in adipose tissue. No association could be found between estimates of dietary SFA intake or the level in adipose tissue with serum cholesterol or LDL-C.

Correlations between proportions of plasma fatty acids and those of dietary intake are summarized in **Table 4.8**. Highest associations were found for PUFA (ρ= 0.162, P= 0.023) and TFA (ρ= 0.165, P= 0.020), while no association could be found with SFA and MUFA intakes. However, a more significant relationship was seen for the P:S ratio (ρ= 0.195, P= 0.006).

Spearman's correlations between plasma fatty acid proportions and those from adipose tissue are illustrated in **Table 4.9**. Significant relationships were seen for total SFA (ρ= 0.145, P= 0.037), MUFA (ρ= 0.378, P= 2.89×10^{-5}) and PUFA (ρ= 0.318, P= 2.66×10^{-6}). No association was found for myristic and stearic acids, but a strong association was found for palmitic acid (ρ= 0.338, P= 5.91×10^{-7}). Palmitoleic (ρ= 0.641, P= 2.26×10^{-25}) and oleic (ρ= 0.264, P= 1.156×10^{-4}) acids were significantly correlated between plasma

and adipose tissue. All PUFA showed a strong significant correlation between plasma and adipose tissue with the exception of adrenic acid (22:4*n*-6).

Table 4.7 Correlations between the proportions of different classes of fatty acid in adipose tissue of female twins compared with estimates from dietary intake (*n*= 570).

	Adipose tissue wt %	Diet % energy	ρ^1	*P*
SFA	25.75±3.02	11.55±2.74	0.084	0.053
MUFA	53.09±3.44	10.50±1.93	-0.006	0.86
PUFA	14.95±2.71	7.21±1.74	0.287	$1.67x10^{-11}$
P:S	0.59 ± 0.15	0.66 ± 0.26	0.331	$5.5x10^{-15}$
TFA	0.53±0.30	0.86±0.38	0.174	$1.85x10^{-4}$

Mean values ± SD for 570 twins.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; TFA: trans-fatty acids.

[1]Spearman's correlation coefficient.

Table 4.8 Correlations between proportions of different classes of fatty acid in plasma of female twins compared with estimates from dietary intake (*n*= 570).

	Plasma wt %	Diet % energy	ρ[1]	*P*
SFA	29.02 ± 2.58	11.55 ± 2.74	0.107	0.131
MUFA	23.43 ± 3.01	10.50 ± 1.93	0.121	0.088
PUFA	41.79 ± 4.66	7.21 ± 1.74	0.162	0.023
P:S	1.45 ± 0.19	0.66 ± 0.26	0.195	0.006
TFA	0.13 ± 0.06	0.86 ± 0.38	0.165	0.020

Mean values ± SD for 570 twins.

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; P:S: ratio of PUFA to SFA TFA: trans-fatty acids.

[1]Spearman's correlation coefficient.

Values for plasma SFA are presented as GM ± SD.

Table 4.9 Correlations between proportions of different classes of fatty acid in plasma of female twins compared with those of adipose tissue.

Fatty Acid	ρ	*P*
SFA	0.145	0.037
14:0	0.116	0.098
16:0	0.338	5.91×10^{-7}
18:0	0.047	0.499
MUFA	0.378	2.89×10^{-5}
16:1*n-7*	0.641	2.26×10^{-25}
18:1*n-9*	0.264	1.15×10^{-4}
18:1*n-7*	0.027	0.718
TFA		
18:1 *trans*	0.349	1.67×10^{-6}
PUFA	0.318	2.66×10^{-6}
n-6	0.323	1.82×10^{-6}
18:2*n-6*	0.385	9.48×10^{-9}
20:3*n-6*	0.401	2.24×10^{-9}
20:4*n-6*	0.209	0.003
22:4*n-6*	-0.041	0.563
n-3	0.293	1.65×10^{-5}
18:3*n-3*	0.406	1.46×10^{-9}
20:5*n-3*	0.318	3.47×10^{-6}
22:5*n-3*	0.205	0.003
22:6*n-3*	0.425	2.09×10^{-10}
n-6:*n-3* ratio	0.362	8.00×10^{-8}
P:S ratio	0.263	1.23×10^{-4}

ρ denotes Spearman's correlation coefficient

4.6.6 Principal component analysis

Data reduction was attempted using principal component analysis on the adipose tissue fatty acids. **Table 4.10** shows the components from the analysis and the scree plot (**Figure 4.6**) generated. Component 1 (high P:S ratio), which explained 22% of the variance, appeared to be determined by higher proportions of LCPUFA and lower proportions of SFA. This is likely to indicate a higher conversion of LA and ALA to LCPUFA or a significant intake of the preformed LC-PUFA. Component 2, which explained 12.28% of the variance, showed a strong correlation with MUFA and AA and negative correlation with SFA and may indicate higher intake of MUFA and active desaturation to AA as its precursor essential fatty acid (EFA) is weakly correlated. Component 3 was found to explain 11.02% of the variance and correlates well with myristic, palmitic and TFA and negatively correlated with capric and lauric acids. Component 4 explained 10.8% of the variance and was associated with higher proportions of palmitic, arachidic and palmitoleic acids, but negatively with EFA, LA and ALA which might suggest that intakes of these fatty acids are very low in addition to their long chain metabolites. The generated components showed no significant differences between MZ and DZ twins as illustrated in **Table 4.11**.

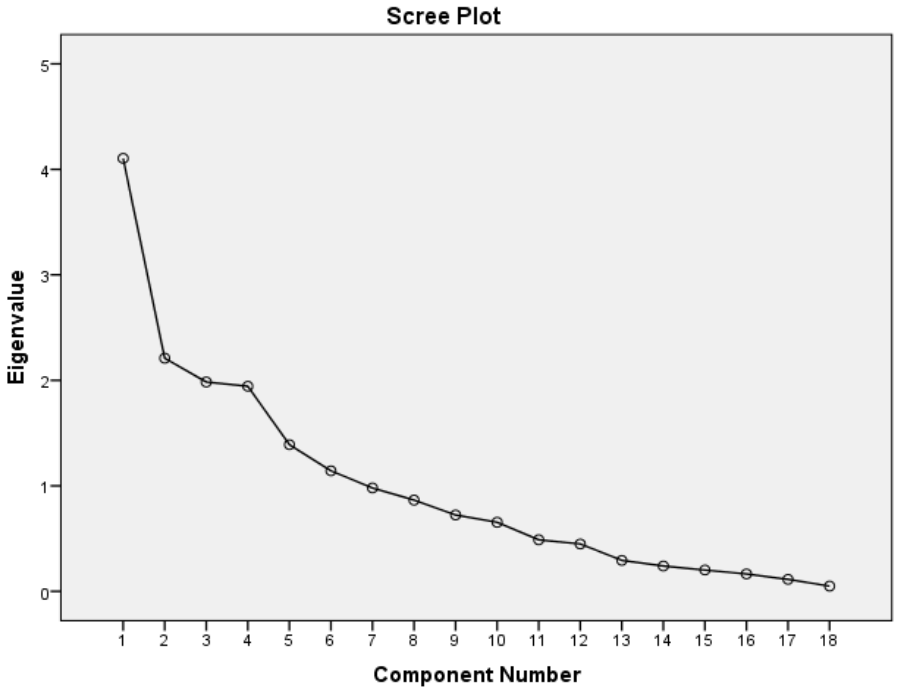


Figure 4.6 Scree plot of the generated components from the application of PCA on the 18 adipose tissue fatty acids

Table 4.10 Association between proportions of 18 adipose tissue fatty acids and 4 identified components.

	Component			
	1	2	3	4
	Low SFA, high LC-PUFA	high MUFA, low SFA	high SFA, high TFA, low PUFA	High SFA, low PUFA
Variance explained (%)	22.8	12.28	11.02	10.8
Eigen value	4.1	2.21	1.98	1.94
10:0	0.016	0.050	**-0.213**	0.113
12:0	**-0.242**	**-0.266**	**-0.785**	0.084
14:0	0.037	-0.137	**0.896**	0.124
16:0	-0.138	**-0.558**	**0.212**	**0.445**
18:0	-0.046	**-0.891**	-0.002	0.075
20:0	**0.195**	-0.072	-0.022	**0.244**
16:1*n*-7	-0.072	**0.739**	0.087	**0.358**
18:1*n*-7	-0.116	**0.215**	-0.147	0.030
18:1*n*-9	-0.065	**0.342**	0.068	-0.045
18:1trans	-0.006	0.103	**0.467**	0.054
18:2*n*-6	0.098	-0.063	-0.100	**-0.862**
20:3*n*-6	**0.684**	0.154	-0.082	0.137
20:4*n*-6	**0.430**	**0.611**	0.062	0.069
22:4*n*-6	**0.802**	0.090	-0.047	0.075
18:3*n*-3	**-0.270**	-0.067	0.042	**-0.777**
20:5*n*-3	**0.861**	-0.040	0.040	0.067
22:5*n*-3	**0.860**	0.123	0.169	-0.018
22:6*n*-3	**0.816**	-0.070	0.170	-0.134

Bold indicates significance $P<0.01$ of correlation.

Table 4.11 Comparison of the generated components from adipose tissue fatty acids between MZ and DZ twins.

Component	MZ	DZ	*P*-value
1	-0.01(-0.15,0.13)	0.01(-0.11,0.13)	0.83
2	-0.03(-0.16,0.10)	0.03(-0.09,0.14)	0.50
3	-0.03(-0.17,0.11)	0.02(-0.10,0.14)	0.60
4	0.07(-0.07,0.21)	-0.05(-0.17,0.07)	0.23
5	-0.02(-0.16,0.12)	0.01(-0.11,0.13)	0.75
6	-0.06(-0.20,0.09)	0.04(-0.08,0.16)	0.31

Univariate analysis of variance was used to perform the comparison.

[1]*P*-value adjusted for age, BMI and ethnicity.

Table 4.12 summarises the generated components from the PCA analysis of plasma fatty acids and scree plot (**Figure 4.7**). Analysis revealed 6 components. Component 1 appeared to reflect a high MUFA+SFA with low LA. Component 2 appeared to reflect the intake of *n*-3 LCPUFA. Component 4 appeared to reflect a higher proportion of *trans* fatty acids. Component 5 might reflect a low P:S ratio and component 6 higher delta-9 desaturation as indicated by stronger positive correlations with 16:1 and 18:1.

Table 4.12 Association between proportions of 18 plasma fatty acids and the 6 identified components.

	Components					
	1	2	3	4	5	6
	High MUFA +SFA, low n-6	Low n-6, high n-3	High stearic + arachidic acids	Low SFA, low EFA	Low P:S	Delta-9 desaturation
Variance (%)	22.48	15.67	11.99	9.40	7.88	6.64
Eigen value	4.05	2.82	2.16	1.69	1.42	1.20
14:0	**0.674**	-0.049	-0.149	**-0.409**	**0.344**	0.120
16:0	**0.694**	-0.080	**-0.253**	-0.075	0.126	-0.041
18:0	0.089	-0.030	**0.302**	**0.272**	**0.683**	-0.088
20:0	-0.044	-0.067	**0.940**	0.053	0.075	-0.032
16:1*n*-7	**0.836**	0.023	-0.036	-0.005	**-0.229**	**0.255**
18:1*n*-9	**0.663**	**-0.289**	-0.035	0.013	**-0.187**	-0.142
18:1*n*-7	**0.347**	0.026	-0.009	**0.320**	**-0.755**	-0.068
18:1*trans*	**0.334**	**-0.341**	-0.037	0.110	**0.175**	**-0.589**
18:2*n*-6	**-0.885**	**-0.240**	0.029	**-0.292**	0.022	-0.076
18:3*n*-6	**0.385**	-0.089	-0.005	**0.215**	**0.372**	**0.604**
20:3*n*-6	**0.193**	**-0.410**	0.038	0.030	-0.027	**0.753**
20:4*n*-6	-0.059	0.160	-0.031	**0.842**	0.065	**0.258**
22:4*n*-6	**-0.182**	-0.029	**0.855**	-0.027	**0.199**	0.122
22:5*n*-6	**-0.374**	**0.269**	**0.536**	**0.187**	**-0.386**	-0.104
18:3*n*-3	-0.043	0.075	-0.075	**-0.649**	0.041	0.109
20:5*n*-3	-0.029	**0.917**	0.048	-0.077	0.014	-0.150
22:5*n*-3	0.074	**0.828**	0.027	0.098	0.049	0.104
22:6*n*-3	-0.126	**0.873**	**-0.113**	0.041	**-0.183**	-0.113

Bold indicates significance $P<0.01$ of correlation.

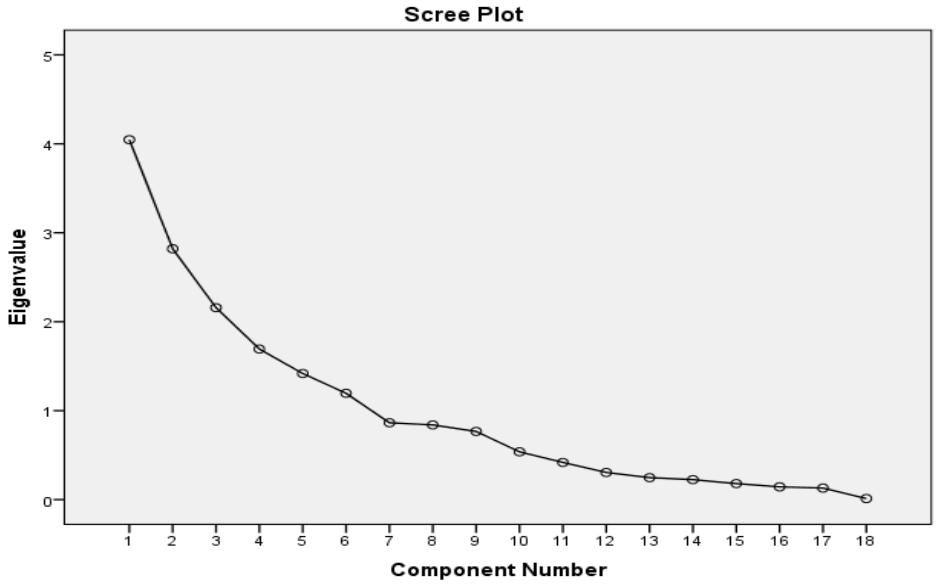


Figure 4.7 Scree plot of the generated components from the application of PCA on the 18 plasma fatty acids.

4.6.7 Results from heritability analysis

The results of genetic modelling analysis for adipose tissue fatty acids are shown in **Table 4.13**. Heritability is estimated based on Falconer'a formula $h^2=2$ (*r* MZ - *r* DZ), where *r* is the correlation coefficient, with adjustment for covariates using structured equation modelling. Model fitting revealed that additive genetic effects (A) could be dropped from the model without significantly deteriorating the Chi-squared goodness-of-fit statistics for all fatty acids except for stearic acid (18:0; 60%) and arachidonic acid (AA; 65%). Most of the variance between subjects in proportions of stearic acid and AA was largely governed by genetic factors. Proportions of the remaining fatty acids appeared to be governed by either contribution from shared (C) or unique (E) environmental factors. Common or shared environmental factors were the main determinant of proportions of lauric, myristic, palmitic and vaccinic acids. Proportions of capric, arachidic, palmitoleic, oleic, TFA, all the *n*-6 and *n*-3 fatty acids, with the exception of arachidonic acid, were manipulated by unique environmental factors (E).

Table 4.13 Genetic modelling analysis for adipose tissue fatty acid composition showing estimates with 95% CI of the proportions of variation within subjects from genetics, common environment and unique environment

	Correlation r (95% CI)		Proportion of variance (95%CI)		
Fatty acid	Monozygotic[1]	Dizygotic[1]	A[2]	C[2]	E[2]
10:0	0.23(0.04,0.40)	0.53(0.40,0.63)	0.00(0.00,0.13)	0.41(0.28,0.51)*	0.59(0.49,0.70)
12:0	0.64(0.51,0.74)	0.67(0.56,0.75)	0.00(0.00,0.18)	0.66(0.50,0.72)*	0.34(0.27,0.42)
14:0	0.65(0.52,0.75)	0.72(0.63,0.79)	0.00(0.00,0.12)	0.69(0.58,0.75)*	0.31(0.25,0.38)
16:0	0.62(0.49,0.72)	0.54(0.39,0.65)	0.20(0.00,0.51)	0.44(0.16,0.64)*	0.36(0.27,0.49)
18:0	0.71(0.59,0.79)	0.34(0.18,0.48)	**0.60(0.27,0.75)***	0.07(0.00,0.35)	0.33(0.25,0.44)
20:0	0.38(0.21,0.53)	0.48(0.33,0.60)	0.00(0.00,0.29)	0.44(0.20,0.54)*	0.56(0.45,0.67)
16:1*n*-7	0.35(0.17,0.51)	0.32(0.17,0.46)	0.00(0.00,0.42)	0.33(0.00,0.44)	0.67(0.52,0.78)
18:1*n*-7	0.79(0.70,0.85)	0.73(0.64,0.80)	0.08(0.00,0.27)	0.70(0.53,0.80)*	0.22(0.16,0.30)
18:1*n*-9	0.41(0.25,0.56)	0.42(0.28,0.54)	0.03(0.00,0.41)	0.39(0.09,0.51)*	0.58(0.44,0.69)
TFA	0.59(0.44,0.71)	0.38(0.22,0.52)	0.32(0.00,0.65)	0.24(0.00,0.53)	0.44(0.33,0.58)
18:2*n*-6	0.51(0.35,0.64)	0.46(0.29,0.59)	0.17(0.00,0.55)	0.37(0.03,0.58)*	0.47(0.35,0.61)
20:3*n*-6	0.52(0.36,0.64)	0.46(0.31,0.58)	0.18(0.00,0.54)	0.36(0.05,0.56)*	0.47(0.35,0.61)
20:4*n*-6	0.64(0.52,0.74)	0.26(0.09,0.40)	**0.65(0.43,0.74)***	0.00(0.00,0.17)	0.35(0.26,0.47)
22:4*n*-6	0.52(0.37,0.64)	0.44(0.28,0.57)	0.20(0.00,0.57)	0.33(0.01,0.55)*	0.47(0.36,0.61)
18:3*n*-3	0.39(0.22,0.54)	0.29(0.11,0.45)	0.30(0.00,0.56)	0.11(0.00,0.41)	0.58(0.44,0.76)
20:5*n*-3	0.39(0.22,0.54)	0.47(0.31,0.60)	0.00(0.00,0.26)	0.43(0.20,0.53)*	0.57(0.46,0.68)
22:5*n*-3	0.54(0.39,0.66)	0.51(0.38,0.62)	0.15(0.00,0.48)	0.41(0.14,0.60)*	0.43(0.33,0.56)
22:6*n*-3	0.37(0.20,0.52)	0.50(0.36,0.61)	0.00(0.00,0.20)	0.44(0.25,0.53)*	0.56(0.47,0.67)

[1]Correlation (95% CI) between subjects in monozygotic twin pairs *n*= 230 and dizygotic twin pairs *n*= 340.

[2]A is Additive genetic, C is common environment, E is unique environment.

Data were analysed using co-twin model adjusted for age and BMI.

*indicate significance (P<0.05).

The intake of SFA is believed to have a significant influence on non-HDL cholesterol (NHDL-C). In order to investigate whether levels of SFA in adipose tissue or the ratio of PUFA:SFA (P/S) in adipose tissue were related to NHDL-C concentration, the following analyses were conducted. ACE modelling was performed with and without adjustment for age, BMI, SFA in adipose tissue and P/S ratio in adipose tissue (**Tables 4.14).** This analysis suggested a strong additive genetic effect on NHDL-C explaining about 45% of the variance. This estimate did not change substantially with adjustment for age, BMI or levels of fatty acids. A regression analysis of NHDL-C with age, BMI, and proportions of SFA and P/S ratio indicated that most of the non-genetic factors could be explained by increasing age and BMI (**Figure 4.8**).

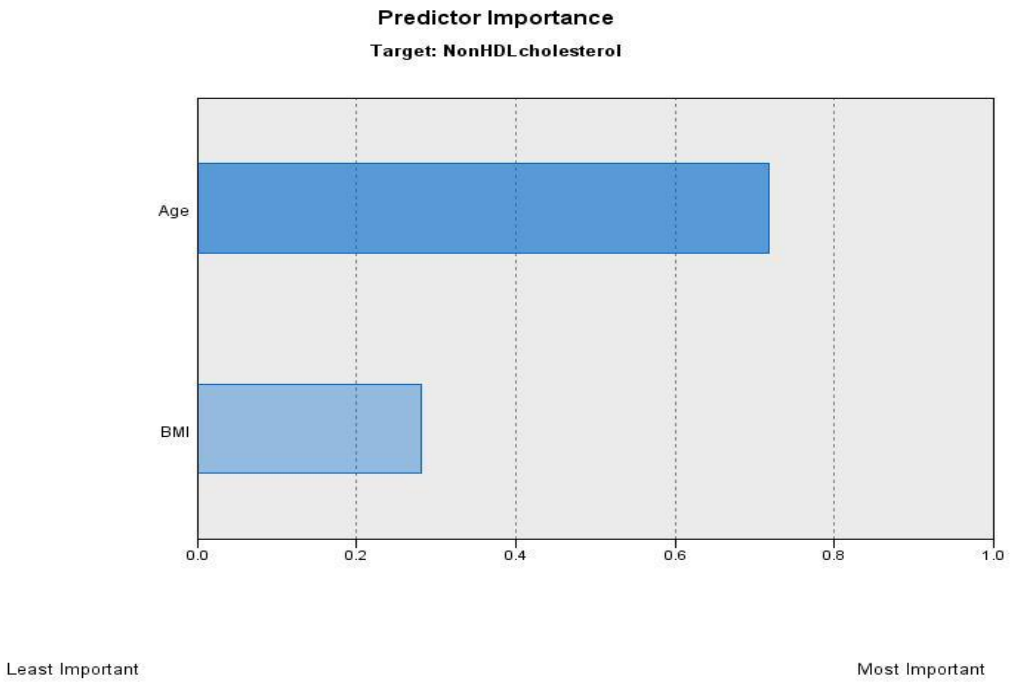


Figure 4.8 Major non-genetic determinants of non-HDL cholesterol in the female twin participants determined by the automated regression analysis module of SPSS.

Table 4.15 summarises genetic modelling analysis for the 6 generated components from adipose tissue fatty acid showing estimates with 95% CI of the proportions of variation within subjects from additive genetics, common environment and unique environmental factors. This indicated a possibly genetic influence for component 2, which was strongly correlated with the proportion of arachidonic acid (AA).

Table 4.14 Genetic modelling analysis for NHDL-C showing estimates with 95% CI of the proportions of variation within subjects from genetics, common environment and unique environment.

	r- Monozygotic[1]	r- Dizygotic[1]	Genetic	Common environment	Unique environment
Non-HDL-C					
Adjusted for age & BMI	0.47(0.31,0.61)	0.17(-0.01,0.34)	0.44(0.06,0.56)*	0.00(0.00,0.30)	0.56(0.44,0.71)
Adjusted for age, BMI & adipose SFA	0.48(0.32,0.61)	0.19(0.01,0.35)	0.45(0.06,0.57)*	0.00(0.00,0.31)	0.55(0.43,0.70)
Adjusted for age, BMI & adipose P:S	0.48(0.32,0.61)	0.19(0.01,0.35)	0.45(0.06,0.57)*	0.00(0.00,0.31)	0.55(0.43,0.70)

[1]Correlation (r) with 95% CI in parenthesis in monozygotic twins *n*= 230 and dizygotic twins *n*= 340.

Data were analysed using co-twin model adjusted for age and BMI.

Table 4.15 Genetic modelling analysis for the 6 generated components from adipose tissue fatty acid showing estimates with 95% CI of the proportions of variation within subjects from genetics, common environment and unique environment.

Component	r- Monozygotic[1]	r- Dizygotic[1]	Genetic	Common environment	Unique environment
Component-1	0.35(0.15,0.51)	0.58(0.41,0.70)	0.00(0.00,0.18)	0.45(0.26,0.56)	0.55(0.44,0.68)
Component-2	0.73(0.61,0.81)	0.45(0.28,0.59)	0.36(0.04,0.73)*	0.32(0.00,0.59)	0.32(0.24,0.43)
Component-3	0.63(0.47,0.74)	0.74(0.64,0.81)	0.00(0.00,0.10)	0.69(0.58,0.75)	0.31(0.25,0.40)
Component-4	0.52(0.34,0.65)	0.44(0.25,0.59)	0.25(0.00,0.64)	0.30(0.00,0.56)	0.45(0.33,0.62)
Component-5	0.73(0.62,0.81)	0.62(0.50,0.72)	0.20(0.00,0.48)	0.52(0.28,0.72)	0.27(0.20,0.38)
Component-6	0.51(0.32,0.66)	0.56(0.42,0.67)	0.00(0.00,0.20)	0.53(0.34,0.62)	0.47(0.38,0.58)

[1]Correlation (r) with 95% CI in parenthesis in monozygotic twins *n*= 230 and dizygotic twins *n*= 340.

Data were analysed using co-twin model adjusted for age and BMI.

The results of genetic modelling analysis for plasma fatty acids are shown in **Table 4.16**. Model fitting revealed that additive genetic effects (A) could not be dropped from the model without significantly deteriorating the Chi-squared goodness-of-fit statistics for all fatty acids except for myristic acid, oleic acid, TFA and DPA. The remaining fatty acids were shown to be manipulated by additive genetic factors (A), which represent the combined effects of alleles at different loci that influence the trait (Bartels *et al*. 2013).

Among SFA, the highest genetic effects appeared to influence proportions of arachidic acid (60%) and a non-significant effect on proportions of palmitic (43%) and stearic (40%) acids. A non-significant genetic effect was also found for palmitoleic (59%) and vaccinic (55%) acids, while variance in proportions of oleic acid (22%) was influenced by minor genetic factors. A finding consistent with our knowledge that oleic acid is derived from both dietary fat and de *novo* lipogenesis. All *n*-6 PUFA were found to be governed by a significant genetic effect (adrenic acid (66%); LA (63%); AA (61%); DGLA (54%); GLA (52%)). Proportion of DHA (65%) and EPA (55%) were the only *n*-3 PUFA that emerged with significant genetic effect. ALA was influenced by 48% genetic effects, but not significant. In contrast, genetic effects explained 21% of the variance in DPA.

Table 4.16 Genetic modelling analysis for plasma fatty acid composition showing estimates with 95% CI of the proportions of variation within subjects from genetics, common environment and unique environment (*n*= 208).

	Correlation r (95% C)		Proportion of variance (95%CI)		
Fatty acid	Monozygotic[1]	Dizygotic[1]	A[2]	C[2]	E[2]
14:0	0.40(0.06,0.65)	0.30(0.05,0.51)	0.27(0.00,0.65)	0.16(0.00,0.50)	0.57(0.35,0.85)*
16:0	0.66(0.43,0.80)	0.44(0.19,0.63)	0.43(0.00,0.78)	0.22(0.00,0.61)	0.34(0.21,0.56)*
18:0	0.50(0.22,0.70)	0.37(0.11,0.57)	0.40(0.00,0.72)	0.15(0.00,0.54)	0.45(0.28,0.73)*
20:0	0.62(0.38,0.78)	0.26(-0.05,0.51)	0.60(0.13,0.75)*	0.00(0.00,0.35)	0.40(0.25,0.63)*
16:1*n*-7	0.51(0.22,0.71)	0.30(0.04,0.52)	0.59(0.00,0.75)	0.00(0.00,0.43)	0.41(0.25,0.70)*
18:1*n*-7	0.58(0.31,0.75)	0.26(0.01,0.49)	0.55(0.00,0.71)	0.00(0.00,0.47)	0.45(0.29,0.70)*
18:1*n*-9	0.60(0.34,0.77)	0.43(0.17,0.63)	0.22(0.00,0.70)	0.33(0.00,0.63)	0.45(0.29,0.67)*
TFA	0.47(0.10,0.71)	0.45(0.18,0.65)	0.16(0.00,0.71)	0.36(0.00,0.63)	0.48(0.28,0.73)*
18:2*n*-6	0.65(0.43,0.80)	0.41(0.14,0.62)	0.63(0.11,0.82)*	0.08(0.00,0.49)	0.29(0.18,0.49)*
18:3*n*-6	0.56(0.30,0.74)	-0.01(-0.33,0.32)	0.52(0.10,0.72)*	0.00(0.00,0.27)	0.48(0.29,0.78)*
20:3*n*-6	0.63(0.39,0.78)	-0.09(-0.42,0.26)	0.54(0.17,0.71)*	0.00(0.00,0.28)	0.46(0.29,0.70)*
20:4*n*-6	0.59(0.34,0.76)	0.28(-0.02,0.52)	0.61(0.08,0.76)*	0.00(0.00,0.40)	0.39(0.24,0.63)*
22:4*n*-6	0.75(0.58,0.85)	0.22(-0.06,0.46)	0.66(0.28,0.78)*	0.00(0.00,0.32)	0.34(0.22,0.51)*
18:3*n*-3	0.55(0.22,0.75)	0.15(-0.13,0.40)	0.48(0.00,0.67)	0.00(0.00,0.47)	0.52(0.33,0.81)*
20:5*n*-3	0.57(0.32,0.74)	0.20(-0.09,0.46)	0.55(0.03,0.71)*	0.00(0.00,0.39)	0.45(0.29,0.69)*
22:5*n*-3	0.54(0.27,0.73)	0.45(0.17,0.65)	0.21(0.00,0.70)	0.33(0.00,0.62)	0.46(0.29,0.70)*
22:6*n*-3	0.75(0.56,0.86)	0.47(0.16,0.67)	0.65(0.19,0.86)*	0.13(0.00,0.52)	0.22(0.14,0.38)*

[1]Correlation (95% CI) between subjects in monozygotic twin pairs *n*= 86 and dizygotic twin pairs *n*= 122.
[2]A is Additive genetic, C is common environment, E is unique environment.
Data were analysed using co-twin model adjusted for age and BMI
*indicate significance (P<0.05).

4.7 Effect of single nucleotide polymorphism in the *FADS1* and *SCD-1* genes on desaturase activities and proportions of LC-PUFA, SFA and MUFA

In order to further investigate the genetic influences on proportions of MUFA and LC-PUFA in adipose tissue and plasma, the rate limiting enzymes in their synthesis, particularly the desaturases, were investigated.

4.7.1 Selection of SNPs

rs174537 (G/T), which is located 14 kb upstream of the *FADS1* gene, is linked with the strongest GWA signal and explains for up to 19% of the differences in plasma arachidonic acid (AA, 20:4*n-6*) (Tanaka *et al*. 2009). Whereas, rs7849 (C/T) in *SCD-1* was found to have a strong association with insulin sensitivity and waist circumference (Warensjö *et al*. 2007)

4.7.2 Statistical analysis

4.7.2.1 SNP allele and genotype frequencies

Two SNPs rs174537 upstream of the *FADS1* and rs7849 in the *SCD-1* gene were genotyped. **Table 4.17** summarises the minor allele and genotype frequencies in all female subjects participanting in the study. Genotype distributions did not deviate from Hardy-Weinberg expectations and minor allele frequencies were in close agreement

with those listed for Europeans on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/, accessed August 2010).

Table 4.17 *FADS1* and *SCD-1* SNP alleles and genotype frequencies.

SNP	Genotype	Allele and genotype frequencies (*n*, %)[1]	*P*[2]
FADS1			
rs174537	MAF	0.34	
	GG	222 (43)	
	GT	238 (46)	
	TT	59 (11)	
	All	519 (100)	0.689
SCD-1			
rs7849	MAF	0.18	
	TT	334 (66)	
	CT	156 (31)	
	CC	14 (3)	
	All	504 (100)	0.404

MAF, minor allele frequency.

[1]Number of subjects for each genotype (% total).

[2] Hardy-Weinberg equilibrium Chi-sq test (1 df).

4.7.2.2 *FADS1* SNP genotype associations with plasma and adipose tissue variables

Table 4.18 shows total LC-PUFA composition and activities of D5D and D6D estimated in adipose tissue stratified by rs174537 genotypes. There were significant associations between SNP genotype and proportions of adipose tissue fatty acids based on a dominant model after adjustment for age and BMI and within pair effects. For related individuals, conventional statistical analyses lead to inflated significance. Dependency of the observations within pairs was accounted for by the use of the Generalized Estimating Equations (GEE) procedure (Trégouët *et al*. 1997) in which both MZ and DZ twins can be used in tests of association. The approach accounts for dependency of the observations within pairs and yields unbiased standard errors and *P*-values.

Carriers of the minor allele had significantly lower proportions of AA (*FADS1* product). Proportions of LA were lower, but not significantly, while those of DGLA were higher in the carriers of the minor allele. In the *n-3* family, significantly lower levels of DPA were found in the carriers of the minor allele and non-significantly lower levels of EPA and DHA were noted in the adipose tissue of the carriers of the minor allele, while proportions of ALA were similar in the two groups. This was illustrated by a significantly lower estimated activity of D5D in the carriers of the minor allele compared to common homozygous.

Table 4.18 Proportions of fatty acids, estimated desaturase activities in adipose tissue stratified by *FADS* SNP genotypes (*n*= 519).

Phenotype	rs174537		
	GG	GT+TT	*P*[1]
	n = 222	*n* = 297	
Fatty acid % total			
18:2*n*-6	12.12 (11.81,12.43)	12.06 (11.79,12.33)	0.937
20:3*n*-6	0.27 (0.26,0.29)	0.28 (0.27,0.30)	0.457
20:4*n*-6	0.59 (0.56,0.61)	0.54 (0.52,0.56)	0.0008
22:4*n*-6[§]	0.23 (0.21,0.25)	0.21(0.20,0.22)	0.088
18:3*n*-3	0.86 (0.83,0.89)	0.86 (0.83,0.88)	0.632
20:5*n*-3[§]	0.19 (0.17,0.20)	0.17 (0.16,0.18)	0.098
22:5*n*-3[§]	0.37 (0.35,0.39)	0.34 (0.32,0.36)	0.006
22:6*n*-3[§]	0.32 (0.30,0.34)	0.30 (0.28,0.32)	0.069
P:S[2]	0.60 (0.58,0.62)	0.59 (0.58,0.61)	0.646
Desaturase activity			
D5D (20:4*n*-6:20:3*n*-6)[2]	2.20 (2.10,2.30)	1.89 (1.81,1.98)	2.59×10^{-6}

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids, mean surrogate estimates of D5D, D6D and D9D activity based on ratio of product:substrate (95% CI). Mean values adjusted for age and BMI.

[1]*P*-value adjusted for within pair effects.

[1]*P*-values corrected for multiple testing.

[§]indicates data were transformed using natural logarithms.

In the plasma, on the other hand, proportions of GLA and AA in the *n*-6 series were significantly lower, whereas the levels of LA and DGLA were significantly higher in the carriers of the minor allele. Non-significantly lower levels of adrenic (22:4*n*-6) and higher levels of osbond (22:5*n*-6) acids were noted. Of the *n*-3 family, significantly lower levels of DPA and DHA were seen in the plasma of the carriers of the minor allele only. D5D and D6D estimated activities were significantly lower in the carriers of the minor allele. Results from the plasma stratified by rs174537 genotypes are shown in **Table 4.19**.

Table 4.19 Proportions of fatty acids (wt%), estimated desaturase activities and lipid concentrations in plasma stratified by *FADS1* SNP genotypes (*n*= 185).

	rs174537		
Phenotype	GG *n* = 66	GT+TT *n* = 119	*P*[1]
18:2*n*-6	25.72 (24.88,26.55)	27.32 (26.70,27.93)	0.0006
18:3*n*-6	0.54 (0.50,0.57)	0.43 (0.40,0.45)	$6.6x10^{-8}$
20:3*n*-6	1.55 (1.46,1.63)	1.66 (1.59,1.72)	0.01
20:4*n*-6	6.95 (6.64,7.25)	5.92 (5.70,6.15)	$2.8x10^{-10}$
22:4*n*-6[§]	0.52 (0.49,0.55)	0.50 (0.48,0.53)	0.561
22:5n-6	1.04 (0.99,1.09)	1.07 (1.03,1.11)	0.596
18:3*n*-3	0.62 (0.58,0.66)	0.63 (0.60,0.66)	0.149
20:5*n*-3[§]	0.19 (0.17,0.20)	0.17 (0.16,0.18)	0.136
22:5*n*-3	0.64 (0.61,0.67)	0.59 (0.57,0.61)	0.002
22:6*n*-3	2.58 (2.39,2.78)	2.43 (2.28,2.57)	0.032
P:S[2]	1.43 (1.39,1.48)	1.46 (1.43,1.49)	0.763
Desaturase activity			
D5D (20:4*n*-6:20:3*n*-6)[2]	4.60 (4.34,4.87)	3.79 (3.59,3.99)	$1.7x10^{-4}$
D6D (18:3*n*-6:18:2*n*-6)[2]	-	-	-
Plasma lipids[2]			
TC	5.61 (5.47,5.75)	5.58 (5.46,5.70)	0.775
HDL-C	1.83 (1.78,1.89)	1.85 (1.80,1.90)	0.585
LDL-C	3.27 (3.14,3.40)	3.21 (3.10,3.33)	0.516
TAG	1.01 (0.99,1.04)	1.01 (0.99,1.03)	0.85
TC:HDL-C ratio	3.21 (3.09,3.33)	3.17 (3.07,3.28)	0.618
NHDL-C	3.78 (3.64,3.92)	3.73 (3.61,3.85)	0.613
Insulin[§]	5.09 (4.93,5.26)	5.20 (5.06,5.35)	0.337
Glucose[§]	2.03 (1.99,2.07)	2.03 (2.00,2.07)	0.891

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids, mean surrogate estimates of D5D, D6D and D9D activity based on ratio of product:substrate (95% CI). Mean values adjusted for age and BMI.

[1]*P*-value adjusted for within pairs effect for MZ and DZ.

[1]*P*-values corrected for multiple testing.

[§] indicates data were transformed using natural logarithms.

4.7.2.3 *SCD-1* SNP genotype associations with plasma and adipose tissue variables

Table 4.20 summarises the proportions of palmitoleic and oleic acids and their precursor fatty acids palmitic and stearic acids, respectively, and activities of D9D desaturases in adipose tissue stratified by rs7849 genotype. There were no significant effects of the SNP on proportions of these fatty acids, but minor allele carriage was associated with slightly higher proportions of palmitic, stearic and palmitoleic acids, while levels of oleic acids were lower than those of the common homozygote.

Table 4.20 Proportions of fatty acids, estimated desaturase activities and lipid concentrations in adipose tissue stratified by *SCD-1* SNP genotypes (*n*= 519).

Phenotype	rs7849		
	TT	TC+CC	*P*[1]
	n = 334	*n* = 170	
Fatty acid % total			
16:0	19.20 (18.98,19.43)	19.25 (18.94,19.56)	0.872
18:0	3.16 (3.07,3.26)	3.27 (3.14,3.40)	0.786
16:1*n*-7	5.80 (5.62,5.98))	5.85 (5.60,6.09)	0.161
18:1*n*-9	46.59 (46.26,46.92)	46.52 (46.05,46.98)	0.807
P:S[2]	0.60 (0.58,0.61)	0.59 (0.57,0.61)	0.560
Desaturase activity[2]			
D9D			
16:1*n*-7:16:0	0.31 (0.30,0.32)	0.31 (0.29,0.32)	0.883
18:1*n*-9:18:0	16.17 (15.64,16.70)	15.82 (15.08,16.56)	0.453

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids, mean surrogate estimates of D5D, D6D and D9D activity based on ratio of product:substrate (95% CI). Mean values adjusted for age and BMI.

[1]*P*-value adjusted for within pair effects.

[1]*P*-values corrected for multiple testing.

In plasma, the SNP in the *SCD-1* gene did not show any significant effects either. Results of the plasma fatty acid proportions stratified by rs7849 genotype are summarised in **Table 4.21**. Minor allele carriage resulted in non-significantly higher levels of palmitic acid and its product, palmitoleic acid. On the other hand, stearic acid proportions were lower and those of its product, oleic acid, were higher in the carriers of the minor allele. This SNP showed no association with the estimated D9D activities, blood lipids levels, glucose, insulin or BMI.

Table 4.21 Proportions of fatty acids (wt %), estimated desaturase activities and lipid concentrations in plasma stratified by *SCD-1* SNP genotypes (*n*= 179).

	rs7849		
Phenotype	TT *n* = 125	TC+CC *n* = 54	*P*[1]
16:0	20.86 (20.62,21.09)	21.26 (20.89,21.63)	0.113
18:0	7.08 (6.98,7.19)	7.02 (6.85,7.18)	0.491
16:1n-7	2.50 (2.39,2.62)	2.55 (2.36,2.73)	0.127
18:1n-9	19.36 (19.01,19.70)	19.91 (19.37,20.45)	0.312
P:S	1.47 (1.43,1.50)	1.41 (1.36,1.46)	0.080
Desaturase activity			
D9D			
16:1*n*-7:16:0	0.12 (0.11,0.12)	0.12 (0.11,0.13)	0.989
18:1n-9:18:0	16.17 (15.64,16.70)	15.82 (15.08,16.56)	0.453
Plasma lipids			
TC	5.59 (5.47,5.70)	5.59 (5.43,5.75)	0.967
HDL-C	1.84 (1.80,1.89)	1.84 (1.78,1.90)	0.968
LDL-C	3.22 (3.12,3.33)	3.25 (3.10,3.40)	0.767
TAG[§]	1.01 (0.99,1.03)	1.00 (0.98,1.03)	0.575
TC:HDL-C ratio	3.20 (3.10,3.30)	3.17 (3.04,3.31)	0.758
NHDL-C	3.74 (3.63,3.86)	3.75 (3.59,3.91)	0.955
Insulin[§]	5.15 (5.01,5.29)	5.21 (5.03,5.41)	0.592
Glucose[§]	2.02 (1.98,2.05)	2.05 (2.01,2.10)	0.236
BMI	5.59 (5.47,5.70)	5.59 (5.43,5.75)	0.967

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids, mean surrogate estimates of D5D, D6D and D9D activity based on ratio of product:substrate (95% CI). Mean values adjusted for age and BMI.
[1]*P*-value adjusted for within pair effects.
P-values corrected for multiple testing.
[§] indicates data were transformed using natural logarithms.

4.8 Discussion

As far as could be ascertained, this is the first study that has compared adipose tissue fatty acid composition between twins. The participants of the study were healthy women. Their dietary intakes and plasma lipid profiles were not unlike those of similar aged women in the National Diet and Nutrition Survey (Henderson *et al*. 2003). Dietary TFA and PUFA intakes showed significant correlations with proportions in adipose tissue. However, the estimated intake of SFA did not correlate with the levels in adipose tissue. Neither was there any association with MUFA intake and the proportion of MUFA in adipose tissue. In the latter case it was noted that the proportion of oleic acid was much higher than estimated in the diet indicating substantial synthesis of this fatty acid. Furthermore, the proportion of palmitic acid was lower and that of palmitoleic was higher which would suggest that a substantial proportion of palmitic acid is converted to palmitoleic acid and that a fraction of palmitic acid is converted to oleic acid. This might explain the lack of association between dietary intake and levels of MUFA and SFA in adipose tissue. A novel finding was the relatively substantial amounts of LC-PUFA in adipose tissue, assuming about 20% of body weight is adipose tissue for a body weight of 68kg this would suggest that out of 13.6 kg, there would be a body pool of about 49g of DHA. Dietary intake of DHA was estimated from the level in plasma to be about 0.13g/d this would equate to about 0.18% of the dietary fatty acid intake. The proportion of DHA in adipose tissue was ~0.36% *vs*. 0.18%, which would imply that at least half of the DHA in adipose tissue was derived from ALA or EPA.

Experimental studies (Mensink *et al*. 2003) have shown that SFA increase TC and particularly LDL-C. NHDL-C concentration is a surrogate measure for LDL-C and is widely used in epidemiological studies in relation to risk of CVD. This cross-sectional study was unable to find any relationship between dietary SFA intake and/or adipose tissue SFA content or that in serum NHDL-C concentration. However, ACE modelling indicated that 45% of the variance could be explained by additive genetics and the remaining variance was explained by environmental effects. Analysis of the non-genetic factors suggests age and BMI were the major determinants of NHDL-C. The range of SFA intakes estimated by the FFQ was not large with SD of about 2.8% energy. Furthermore, only 60-70% of the SFA intake is likely to be C12-C16 SFA and consequently the difference between 1 SD either side of the mean would only be about 3.5% energy. Using the Keys equation, a 3.5% energy difference in SFA would predict a difference in serum cholesterol of a 0.2 mmol/L, which is below 5% of the variance and may be too small an effect to detect in a cross-sectional study. This failure to find a relationship between SFA intake and NHDL-C is consistent with the findings of the UK NDNS and most other reports from cohort studies. However, where differences in SFAs are large for example when comparing vegans with omnivores, it is possible in cross-sectional studies to show a relationship with NHDL-C (Crowe *et al*. 2013).

The present study aimed to distinguish the possible effect of genetic factors from those of the environment, represented by dietary consumption and food choices, on proportions of adipose tissue fatty acids in MZ and DZ female twins. Genetic modelling

analysis of data from 570 participants demonstrated that 60% and 63% of variation in the proportions of stearic acid and AA, respectively, was attributable to genetic factors.

Two main factors determine variation of fatty acid proportions in adipose tissue. These are dietary intake and endogenous synthesis/remodelling, which involves enzymatic elongation and desaturation of precursor fatty acids. Both of these factors have been shown to have some degree of genetic control. Teucher *et al*. (2007) showed that food choice or dietary pattern is governed by genetic factors to some extent after analysis of the dietary intake of twin subjects. Endogenous synthesis of fatty acids depends on activities of enzymes subject to genetic variation. The high heritability of stearic acid may indicate variability of conversion to oleic acid by delta-9 desaturase. LA cannot be synthesised *de novo* but is converted to AA. Small amounts of preformed AA are present in meat and eggs but the amounts are very much smaller than the amounts of EPA and DHA provided by oily fish. It is likely, therefore that the higher amount is a consequence of differences in its synthesis from LA. The high heritability of AA was also confirmed by the plasma analysis.

This work was then able to demonstrate that SNP rs174537 upstream of the *FADS1* gene is associated with proportions of AA in adipose tissue and plasma. On the other hand, SNP rs7849 in the *SCD-1* gene was found to be associate with proportions of palmitic and stearic acids and their metabolites, palmitoleic and oleic acids, in plasma and adipose tissue, but not significantly. This might be due to the fact that the *SCD-1* gene is expressed in the liver and not in adipose tissue or that there is a different variant

of *SCD*-1 expressed in adipose tissue. Furthermore, the relatively small sample size has limitations with regard to power to detect significant differences.

4.9 Conclusion

In conclusion, the present study demonstrated that PUFA and TFA in both adipose tissue and plasma correlate and are good biomarkers of dietary intake but the proportions of SFA in adipose tissue are weak biomarkers of SFA intake. This work also revealed that adipose tissue is a significant reservoir of DHA and other LC-PUFA. It was demonstrated that it is possible to estimate intakes of EPA and DHA from the proportions in plasma. A significant additive genetic effect on the proportion of AA in adipose tissue and of AA in plasma was detected. Some of this variation could be explained by polymorphism rs174537 upstream of the *FADS1* gene.

Chapter 5: MARINA study: Influence of genetic variation on plasma and erythrocyte lipid proportions in response to EPA and DHA intake

5.1 Introduction

EPA and DHA play a crucial role at cellular level. In addition to maintaining cell membrane fluidity and integrity, they are of importance for retinal and neurological functions. Phosphlipids in the brain and retina contain 20-50% DHA. EPA and DHA can also act as ligands for PPARs and influence transcriptional events at a molecular level. For these reasons, consumption of dietary sources rich in these two fatty acids might be anti-inflammatory, cardio-protective and reduce risk to CVD (Burdge, 2004).

While the essentiality of LA and its metabolites are well established, the physiological role of the *n*-3 series is less certain. The essentiality of the *n*-3 series appears to hinge on the conversion of ALA to DHA. Requirements for *n*-3 LC-PUFA can be probably met by ALA in most species including primates and studies on human infants have shown active conversion of ALA to DHA (Jensen *et al*. 1997). However, high intakes of LA may inhibit this conversion by competitive inhibition of delta-6 desaturase (D6D). Soybean oil and rapeseed oil are the chief dietary sources of ALA although milk fat (provides 1-1.5%) and walnuts are rich sources. ALA can be converted into EPA and DHA by alternating desaturation and chain elongation reactions. The liver is a major site of conversion (Burdge & Calder, 2005), but there is evidence that conversion takes place inside the brain (Sanders *et al*. 1984) and other tissues such as the testis (Leat *et al*. 1983). In the land-based food chain, the formation of DHA is dependent upon conversion from ALA and this is also influenced by the relative amount of LA in the diet. Small amounts of *n*-3 LC-PUFA, mainly DHA, are present in animal products,

especially offal, but including meat and eggs. However, the amount present in most human diets is small (50-100mg) and usually no more than 200mg/d. In the marine food chain significant amounts of preformed EPA and DHA are present especially in oily fish such as herring, mackerel, sardines, salmon and tuna. These fatty acids are derived from algae which make these fatty acids mainly by the polyketide synthase pathways (Metz *et al*. 2001). The consumption of EPA and DHA has a far more marked effect on the proportions of these fatty acids in plasma and membrane lipids than dietary ALA (Sanders, 2009a). Minimal requirements for *n*-3 LC-PUFA are in the range of 0.2%-0.5% of the total energy intake and it is generally accepted that this requirement can be met by ALA. Some recent dietary guidelines have suggested a population intake of 0.25g *n*-3 LC-PUFA (WHO/FAO 2010; EFSA 2010) because of the association of fish intake with a lower risk of CVD. However, no recommendations for intakes of *n*-3 LC-PUFA were made in the Dietary Guidelines for Americans 2010 which were made on the basis of a meticulous systematic review of the evidence (http://www.nutritionevidencelibrary.gov)

EPA and DHA are preferentially esterified into the *sn*-2 position of phospholipids (Flachs *et al*. 2009; Kopecky *et al*. 2009). Different phospholipids classes have specificity for different PUFA. For instance, AA is the dominant PUFA in phosphatidylinositol, while DHA is the principal fatty acid in retinal ethanolamine phosphoglycerides. Phospholipids, above and beyond being essential membrane components, operate as a substrate for chemical messengers, such as eicosanoids C_{20}

LC-PUFA (Flachs *et al*. 2009). AA is the usual substrate for cyclo-oxygenases type 1 and 2 (COX-1 and COX-2). EPA forms a series of metabolites that are generally inactive or less active than those derivatives of AA. DHA does not give rise to prostaglandins but inhibits the formation of prostaglandins derived from AA in a similar manner with EPA. More recently, metabolites called neuroprotectins and resolvins have been shown to be from EPA and DHA (Serhan *et al*. 2004). Eicosanoids derived from *n*-6 PUFA are vital for normal body functions and are usually synthesised by COX-1 but extreme eicosanoid formation can be induced as a result of inflammation and injury via activation of COX-2 (Flachs *et al*. 2009; Kopecky *et al*. 2009; Sanders & Emery, 2003). The replacement of AA with EPA and DHA can modulate inflammation. However, if EPA replaces too much AA it results in pathological changes. For example, parturition is inhibited (Sanders, 1988). The extent to which ALA is converted to EPA and DHA has been a topic of much debate. Groups of people such as vegans who have no dietary intakes of EPA and DHA have levels of EPA and DHA about 2/3 lower than that of omnivores (Sanders *et al*. 1978). Supplementation studies in adults have shown that ALA is converted into EPA but without influencing DHA (Sanderson *et al*. 2002). However, studies conducted on infants show that ALA is converted to DHA. There have been reports (Welch *et al*. 2010) suggesting that women may be more able to convent ALA to DHA. It has also been suggested that the capacity to desaturase besides being under hormonal control may also be influenced by genetic factors (Schaeffer *et al*. 2006). Plasma lipids are believed to reflect short term changes in fatty acid intake whereas erythrocyte lipids are believed to be a better marker of tissue status.

This chapter reports on the effects of different doses of EPA and DHA on levels of these fatty acids in plasma and erythrocytes and the effects of polymorphisms in the *FADS1 FADS2* gene cluster on proportions of LC-PUFA based on the analysis of samples from the MARINA study.

5.2 Purpose of the study

Dietary intake of fish is associated with a lower risk to CVD. It is thought that this protective effect is mediated, in part, by the presence of long-chain *n*-3 PUFA (LC-*n-3*-PUFA) in fish particularly eicosapentaenoic (20:5*n-3*; EPA) and docosahexaenoic acids (22:6*n-3*; DHA). Levels of EPA and DHA in blood are strongly influenced by the consumption of dietary sources rich in these fatty acid, but may also be influenced by the intakes of linoleic (18:2*n-6*; LA) and α-linolenic (18:3*n-3*; ALA) acids and genetic factors. The dose-response relationship of increasing intakes of LC *n-3* PUFA on levels in plasma and erythrocytes have been examined in short term studies but there is a lack of information from longer term studies of six months duration or longer.

The aims were

1. To investigate the influence of single nucleotide polymorphism in the *FADS1-FADS2* gene cluster on levels of PUFA and LC-PUFA in erythrocyte phospholipids and total plasma fatty acids at baseline and after 6 months of graded intake of EPA+DHA (ratio of 1.51:1).

2. Develop equations to predict intakes of EPA and DHA from analysis of plasma lipids in other studies.

3. To identify possible post-intervention novel circulating fatty acid pattern using principal component analysis as a modelling technique.

4. To investigate whether the measures of *n*-3 PUFA in the participants at baseline is associated with *in vivo* measures of atherosclerosis determined by carotid intimal media thickness.

The following objectives were set to meet the above aims:

1. To measure fatty acid proportions in both erythrocyte phospholipids and total plasma at baseline and after 6-months following supplementation of graded intakes of EPA + DHA equivalent to intakes of 1, 2 or 4 servings of oily fish per week by gas chromatography.

2. To perform PCA analysis on proportions of fatty acids after 6 months intervention to identify circulating pattern of fatty acids.

3. To investigate whether the fatty acid profile is associated with atherosclerosis.

Hypothesis (1)

Supplementation of graded intake of EPA + DHA for 6-months will modify fatty acid proportions of plasma and erythrocyte phospholipids in a linear dose dependent manner

Hypothesis (2)

Single nucleotide polymorphism in the *FADS1-FADS2* gene cluster influences the proportions of parent essential fatty acid and their LC-PUFA in the erythrocyte phospholipids and total plasma fatty acids at baseline and after 6 months of consumption of EPA+DHA.

5.3 Study participants and design

The MARINA (Modulation of Atherosclerosis Risk by Increasing doses of N-3 fatty Acids) trial was a single-centre dietary intervention study of randomized double-blind parallel design, to test the effects of three daily doses of EPA and DHA on endothelial function and established CVD risk factors (Sanders *et al*. 2011). The study was approved by the St Thomas' Hospital NHS Research Ethics Committee (NREC 08/H0802/3) and written informed consent was given by participants, who were healthy non-smoking men and women aged between 45-70y, recruited through media advertisements and screened as described previously (Sanders *et al*. 2011). A total of 367 participants were randomized to treatment by computer-generated sequence, using the process of minimization to balance age, gender and ethnicity between treatment groups. During an initial run-in period of 4 weeks, participants took olive oil (BP specification) placebo capsules whilst restricting oily fish intake, after which baseline measurements of outcome variables were made. The dietary intervention phase involved supplementation with encapsulated EPA and DHA at three doses (0.45, 0.9 and 1.8 g/day), compared with placebo. The dose range was selected to reflect the range of intakes likely to be encountered in human diet rather than extreme diets (SACN Advice of Fish Consumption 2004). Current UK dietary advice is to consume two portions of fish a week one of which is oily. The present investigation is based on measurements made at baseline and after 6 months. Compliance was determined by assessing the EPA and DHA content in erythrocyte phosphoglycerides at baseline, 6 and 12 months. The

participants were supplied with capsules at regular intervals and any unused were returned and their numbers recorded. The oil blends were supplied by Croda Chemicals Europe Ltd. (Hull, UK) and encapsulated in gelatine by Powerhealth (Pocklington, UK) as described previously (Sanders *et al*. 2011). Quality control analysis was performed by Croda Chemicals Europe Ltd.

5.4 Blood sampling and analysis

Erythrocyte lipids were extracted from washed cells within 3 days of blood collection as described in Chapter 2. Extracted lipids were stored at -20°C until analyzed. Blood samples for analysis were drawn after a minimum 8hrs overnight fast preceded by a low-fat evening meal (<10g fat, 3MJ) and serum was stored at -45°C until analyzed. Plasma total fatty acids were determined by capillary gas-liquid chromatography (GLC) as described in Chapter 2, substituting toluene for benzene and using pentadecanoic acid as an internal standard (Lepage & Roy, 1986). Measurements of plasma lipid concentrations were as described in Chapter 2 and inter- assay coefficients of variation were as previously reported (Sanders *et al.* 2011). Surrogate estimates of desaturase activity was based on product:substrate ratios of *n*-6 LC-PUFA measures available. AA:DGLA (20:4*n-6*:20:3*n-6*) was used to estimate D5D activity (*FADS1*) and GLA:LA (18:3*n-6*:18:2*n-6*) for D6D (*FADS2*) as reported in other studies.

5.5 Carotid intima-medial thickness (CIMT) assessed by high resolution ultrasound

It is possible to assess the extent of atherosclerosis in vivo by measuring carotid intima medial thickness. With increase age, the intima thickens and this represents a measure of atherosclerosis. It can be measured by high-resolution ultrasound non-invasively and is regarded as a gold standard method for assessing atherosclerosis in healthy subjects. CIMT changes very slowly so value measured at the end of the study are likely to be similar to those at baseline.

5.6 Selection and analysis of SNPs

Selected SNPs were sited within *FADS1* and *FADS2* genes or potential 5' or 3' regulatory regions and in strong LD to enable haplotype analysis (r^2>0.8). High minor allele frequencies (>10%) are required for investigation of diet-gene interaction. The rs174537 (G/T) located 14 kb upstream of the *FADS1* gene is associated with the strongest GWA signal and accounts for up to 19% of the variation in plasma arachidonic acid (AA, 20:4*n*-*6*) (Tanaka *et al*. 2009). rs174561 (T/C) is located in intron 1 of *FADS1* and rs3834458 (T/del) is located in the promoter region of *FADS2*.

5.6.1 DNA extraction and SNP genotyping

Buffy coats removed from blood samples were stored in EDTA at -20°C. Genomic DNA was extracted from 200µl using an Illustra blood genomic prep mini spin kit (GE

Healthcare, Amersham, UK) according to manufacturer's instructions. Genotyping was performed on the 310 participants for whom DNA was available by KBiosciences (Hoddesdon, UK), using the KASPar system. Genotype accuracy, as assessed by inclusion of duplicates in the array was 98% and negative controls (water blanks) were included on each plate. The mean genotyping success rate was 97.7% (95.8-99.7%).

5.6.2 Haplotype analysis

Haplotype analysis was performed using the graphical JAVA interface of the THESIAS software package (Tregouet & Garelle, 2007), (available online at http://ecgene.net/genecanvas). This program is based on the maximum likelihood model linked to the SEM algorithm (Tregouet *et al*. 2004) and used to statistically reconstruct haplotypes in unrelated individuals and perform haplotype-based association analysis of phenotypes. Covariate-adjusted haplotype effects as well as interactions between haplotypes and covariates can be investigated.

5.7 Statistical analysis

All genotype distributions were tested for deviation from Hardy-Weinberg equilibrium using a $\chi 2$ test with 1 df ($P > 0.05$). Inter-locus linkage disequilibrium based on observed numbers of diplotypes was established using CubeX software (Gaunt *et al*. 2007), (available online at http://www.oege.org/software/cubex/) and represented in a correlation matrix as Lewontin's D′ and squared correlation r^2 measures between each pair of SNP loci.

Statistical analyses were carried out using the SPSS version 17.0 for Windows (SPSS Inc, Chicago, IL, USA). Normal distribution of outcome variables was evaluated by Q-Q plots. Where needed, variables were log transformed to obtain better approximations of the normal distribution prior to analysis. Due to the limited sample size, especially after stratification for dietary intake, SNP genotype association analyses were based on a dominant inheritance model. The presence of any significant difference in three SNP genotype frequencies between the four treatment groups was ascertained by $\chi 2$ test with 6 df ($P > 0.05$). Linear regression was used to assess independent SNP and haplotype associations with phenotypes and interaction with dietary treatment. All data presented in text and tables are expressed as means or geometric means ± standard deviation (SD) or mean (95% CI). Ethnicity, sex, age and body mass index (BMI) were added to the models as covariates to adjust for possible confounding effects. Interaction between *FADS* SNP genotype and dosage of EPA+DHA was explored by adding interaction terms to the multiple linear regression models. Association between proportions of LC-PUFAs in plasma and erythrocyte phosphoglycerides was evaluated by Spearman's correlation coefficient (ρ) with significance set at $P < 0.01$ (two-tailed test). Multivariate analysis of variance was used to allow for multiple testing of genotype associations with proportions of nine fatty acids and univariate analysis of variance to test genotype associations with surrogate measures of desaturase activities without adjustment for multiple comparisons. Significance was taken as $P < 0.05$ (as confirmed in the multivariate analysis of variance).

5.8 Results

5.8.1 Characteristics of subjects

Data was available for analysis of 310 out of 367 participants randomized to treatment. The number of participants allocated and drop-out rates did not differ significantly between treatment groups (Sanders *et al*. 2011). There were more women than men approximately 1.6:1 and ~20% of the sample was non-white, with similar proportions of Asian and Black participants. The average BMI was above the desirable range (20-25 kg/m^2) and the mean waist circumferences were greater than cut-offs indicating risk of metabolic syndrome (94 cm in men and 80 cm in women) (Sanders *et al*. 2011). **Table 5.1** shows the details of subjects after 4 week run-in on placebo, for whom DNA samples were available, and the PUFA in plasma and erythrocyte lipids. The proportion of LA was much higher in plasma than erythrocytes reflecting the higher proportions in cholesteryl esters and lecithin. There were no significant differences in these measures between the four treatment groups at baseline ($P > 0.05$).

Table 5.1 Characteristics of the sample.

	Placebo	0.45 g/day	0.9 g/day	1.8 g/day
	n = 71	*n* = 82	*n* = 81	*n* = 82
Male *n* (%)	31 (35)	31 (38)	30 (37)	31 (38)
Female *n* (%)	42 (48)	51 (62)	51 (63)	51 (62)
Age (y)	55.37±6.96	55.00±6.79	55.16±6.56	55.02±6.65
BMI (kg/m^2)	26.24±3.72	25.12±3.86	26.13±4	25.22±3.49
Plasma fatty acid % total				
Linoleic acid (18:2*n-6*)	26.99±4.02	27.54±3.62	27.69±4.14	27.83±3.91
γ-linolenic acid (18:3*n-6*)	0.47±0.16	0.43±0.18	0.45±0.16	0.46±0.18
Dihomo-γ-linolenic acid (20:3*n-6*)	1.62±0.37	1.58±0.32	1.59±0.29	1.63±0.36
Arachidonic acid (20:4*n-6*)	6.91±1.61	6.74±1.43	7.12±1.69	6.84±1.36
Adrenic acid (22:4*n-6*)	0.60±0.15	0.57±0.13	0.61±0.14	0.60±0.14
α-linolenic acid (18:3*n-3*)	0.67±0.25	0.61±0.17	0.66±0.20	0.64±0.20
Eicosapentaenoic acid (20:5*n-3*)	1.22±0.74	1.10±0.60	1.12±0.57	1.10±0.56
Docosapentaenoic acid (22:5*n-3*)	0.66±0.12	0.64±0.12	0.64±0.13	0.65±0.13
Docosahexaenoic acid (22:6*n-3*)	2.40±0.79	2.42±0.78	2.36±0.67	2.45±0.68
Plasma desaturase activity				
D5D (20:4n-6:20:3*n-6*)	4.47±1.33	4.44±1.32	4.64±1.46	4.39±1.28
D6D (18:3n-6:18:2*n-6*)	0.17±0.01	0.16±0.01	0.16±0.01	0.16±0.01
Erythrocyte fatty acid % total				
Linoleic acid (18:2*n-6*)	10.84±1.56	11.12±1.45	11.00±1.42	11.00±1.57
γ-linolenic acid (18:3*n-6*)	-	-	-	-
Dihomo-γ-linolenic acid (20:3*n-6*)	1.90±0.37	1.90±0.38	1.83±0.38	1.89±0.40
Arachidonic acid (20:4*n-6*)	16.76±1.96	16.37±2.12	16.92±2.12	16.44±2.03
Adrenic acid (22:4*n-6*)	2.78±1.01	2.91±1.03	2.99±2.18	2.79±0.66
α-linolenic acid (18:3*n-3*)	0.19±0.12	0.20±0.20	0.18±0.10	0.18±0.13
Eicosapentaenoic acid (20:5*n-3*)	1.30±0.47	1.26±0.46	1.36±0.55	1.31±0.50
Docosapentaenoic acid (22:5*n-3*)	3.23±0.40	3.20±0.53	3.23±0.55	3.22±0.56
Docosahexaenoic acid (22:6*n-3*)	6.52±1.57	6.47±1.34	6.32±1.50	6.48±1.51
Erythrocyte desaturase activity				
D5D (20:4*n-6*:20:3*n-6*)	9.14±1.91	8.91±1.87	9.41±2.08	9.01±1.88
D6D (18:3*n-6*:18:2*n-6*)	-	-	-	-

Measurements made at baseline after 4-wk run-in on normal diet with placebo supplement are shown for each randomized EPA+DHA treatment group. Values are *n* (%) for Male/Female or mean ± SD for all other variables and do not differ significantly by treatment allocation (P>0.05).

5.8.2 SNP allele and genotype frequencies

Three SNPs at the *FADS1-FADS2* gene locus, rs174537, rs174561 and rs3834458 were genotyped. The minor allele and genotype frequencies in all subjects who completed the study (*n*= 310) are shown in **Table 5.2**. Genotype distributions did not deviate from Hardy- Weinberg expectations and minor allele frequencies were in close agreement with those listed for Europeans on the NCBI SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/ [accessed August 2010].

Table 5.2 *FADS1* and *FADS2* SNP allele and genotype frequencies.

SNP	Genotype	Allele and genotype frequencies (*n*, %)[a]	*P*[b]
rs174537	MAF	0.30	
	GG	153 (45)	
	GT	126 (37)	
	TT	30 (9)	
	All	309 (100)	0.58
rs174561	MAF	0.27	
	TT	162 (47)	
	TC	116 (34)	
	CC	25 (7)	
	All	303 (100)	0.51
rs3834458	MAF	0.30	
	TT	151 (44)	
	T/-	115 (34)	
	-/-	31 (9)	
	All	297 (100)	0.19

MAF, minor allele frequency.
[a]No. of subjects for each genotype (% total).
[b]Hardy-Weinberg equilibrium Chi-sq test (1 df).

5.8.3 SNP genotype associations with plasma and erythrocyte variables at baseline

Erythrocyte measures previously confirmed establishment of similar levels of *n*-3 LC-PUFA in all treatment groups during the run-in on placebo and compliance over the intervention period (Sanders *et al*. 2011). Proportions of fatty acids in plasma and erythrocyte phosphoglycerides were highly correlated ($P < 0.01$), except for adrenic acid (22:4*n-6*), which is rapidly taken up from plasma by erythrocytes (**Table 5.3**).

Table 5.3 Spearman's correlation between proportions of fatty acids and indices of D5D activity estimations in plasma and erythrocytes at baseline

	Plasma	Erythrocyte	ρ
Fatty acid % total[a]			
18:2*n-6*	27.50 ± 3.95	11.01 ± 1.49	0.614[b]
18:3*n-6*	0.46 ± 0.17	-	-
20:3*n-6*	1.61 ± 0.33	1.88 ± 0.39	0.564[b]
20:4*n-6*	6.89 ± 1.54	16.54 ± 2.12	0.385[b]
22:4*n-6*	0.58 ± 0.15	2.88 ± 1.34	-0.046
18:3*n-3*	0.65 ± 0.20	0.19 ± 0.14	0.398[b]
20:5*n-3*	1.13 ± 0.60	1.30 ± 0.50	0.628[b]
22:5*n-3*	0.64 ± 0.12	3.22 ± 0.53	0.411[b]
22:6*n-3*	2.41 ± 0.73	6.40 ± 1.51	0.631[b]
Desaturaseactivity[a]			
D5D[c]	4.46 ± 1.35	9.74 ± 12.60	0.652[b]

[a]Mean ± SD.

[b]Correlation significant at the $P = 0.01$ level (2-tailed).

[c]Activity measured by 20:4*n-6*:20:3*n-6* ratio.

Table 5.4 shows total LC-PUFA composition and activities of D5D and D6D estimated in plasma stratified by rs174537, rs174561 and rs3834458 genotypes, after a 4-week run-in on normal diet with placebo supplement. There were significant associations between SNP genotypes and proportions of plasma fatty acids based on a dominant model after adjustment for ethnicity, gender, age and BMI. In the *n*-6 series, the minor alleles of all SNPs were associated with a higher proportion of *FADS1* substrate dihomo γ-linolenic acid (DGLA, 20:3*n-6*) and lower proportions of *FADS2* product γ-linolenic acid (GLA, 18:3*n-6*), *FADS1* product AA (20:4*n-6*) and its derivative adrenic acid. In the *n*-3 family, minor alleles were significantly associated with higher proportion of *FADS1* substrate ALA (18:3*n*-3) and lower proportions of *FADS2* product EPA (20:5*n*-3) and its derivatives docosapentaenoic acid (DPA, 22:5*n-3*) and DHA (22:6*n-3*). Increased substrate and decreased downstream product proportions inferred a reduction in desaturase activities. In line with these observations, carriers of the minor alleles of all three SNPs showed highly significant associations with lower estimated activities of D5D and D6D compared to common homozygotes.

Table 5.4 Proportions of fatty acids and estimated indices of desaturase activities in plasma stratified by *FADS1-FADS2* SNP genotype at baseline.

Phenotype	rs174537			rs174561			rs3834458		
	GG	GT+TT	*P*[a]	TT	TC+CC	*P*	TT	Tdel + deldel	*P*
	n = 151	*n* = 125		*n* = 160	*n* = 140		*n* = 160	*n* = 144	
18:2*n-6*	27.2 (26.5,27.8)	27.6 (26.9,28.2)	0.17	26.5 (25.6,27.4)	27.9 (27.2,28.5)	0.016	26.9 (26.0,27.9)	27.6 (26.9,28.2)	0.26
18:3*n-6*	0.5 (0.5,0.5)	0.4 (0.4,0.4)	1.90×10^{-8}	0.5 (0.5,0.6)	0.4 (0.4,0.4)	4.56×10^{-8}	0.5 (0.5,0.6)	0.41 (0.39,0.44)	4.96×10^{-8}
20:3*n-6*	1.5 (1.5,1.6)	1.7 (1.7,1.8)	3.85×10^{-6}	1.5 (1.5,1.6)	1.7 (1.7,1.8)	5.29×10^{-6}	1.6 (1.5,1.6)	1.70 (1.65,1.75)	1.52×10^{-5}
20:4*n-6*	7.6 (7.4,7.8)	6.3 (6.1,6.5)	7.56×10^{-16}	7.5 (7.2,7.9)	6.2 (5.9,6.4)	2.49×10^{-16}	7.5 (7.2,7.9)	6.24 (6.01,6.47)	3.83×10^{-16}
22:4*n-6*	0.6 (0.6,0.6)	0.6 (0.5,0.6)	0.007	0.6 (0.6,0.6)	0.6 (0.5,0.6)	0.05	0.6 (0.6,0.6)	0.56 (0.54,0.58)	0.002
18:3*n-3*	0.6 (0.6,0.7)	0.7 (0.6,0.7)	0.13	0.6 (0.6,0.7)	0.7 (0.6,0.7)	0.018	0.6 (0.6,0.7)	0.67 (0.64,0.70)	0.008
20:5*n-3*	1.2 (1.1,1.3)	1.02 (0.9,1.1)	0.003	1.1 (0.9,1.2)	1.01 (0.9,1.1)	0.004	1.1 (0.9,1.2)	1.03 (0.93,1.12)	0.023
22:5*n-3*	0.7 (0.7,0.7)	0.6 (0.6,0.6)	4.47×10^{-7}	0.7 (0.6,0.7)	0.6 (0.6,0.6)	2.69×10^{-7}	0.7 (0.7,0.7)	0.61 (0.59,0.63)	3.14×10^{-5}
22:6*n-3*	2.5 (2.4,2.6)	2.3 (2.2,2.4)	0.001	2.3 (2.2,2.5)	2.2 (2.1,2.4)	2.18×10^{-4}	2.3 (2.2,2.5)	2.27 (2.15,2.38)	0.003
D5D[b]	5.1 (4.9,5.3)	3.8 (3.6,4.0)	1.38×10^{-18}	5.1 (4.9,5.3)	3.8 (3.6,3.9)	5.84×10^{-19}	5.1 (4.9,5.3)	3.82 (3.62,4.01)	4.15×10^{-18}
D6D[c]	0.18 (0.17,0.18)	0.16 (0.15,0.16)	3.26×10^{-7}	0.18 (0.17,0.18)	0.16 (0.15,0.16)	6.05×10^{-8}	0.17 (0.17,0.18)	0.16 (0.15,0.16)	4.20×10^{-7}

Mean values (95% CI) estimates of D5D and D6D activity based on ratio of *n-6* product:substrate (95% CI).

[a]Genotype association with fatty acids and desaturase activitieswas tested respectively by multivariate and univariate analysis of variance based on a dominant model. *P*-values adjusted for BMI, age, gender and ethnicity.

[b]Activity estimated by 20:4*n-6*:20:3*n-6* ratio.

[c]Activity estimated by 18:3*n-6*:18:2*n-6* ratio.

In erythrocytes, DGLA was the only LC-PUFA showing associations with genotypes with significance comparable to those in plasma ($2.97 \times 10^{-19} \leq P \leq 6.03 \times 10^{-15}$). D5D activity estimated in erythrocytes was much higher than in plasma, a reflection of the higher proportion of AA (16.5% versus 6.9%), but all minor alleles were associated with significantly lower activity ($1.60 \times 10^{-21} \leq P \leq 2.68 \times 10^{-16}$). D6D activity was not estimated in erythrocytes, as GLA was not detected. Erythrocyte data are presented in **Table 5.5.**

Table 5.5 Proportions of fatty acids and indices of desaturase activities in erythrocytes stratified by *FADS1-FADS2* SNP genotype at baseline.

Phenotype	rs174537			rs174561			rs3834458		
	GG	GT+TT	*P*[a]	TT	TC+CC	*P*	TT	Tdel + deldel	*P*
	n = 147	*n* = 151		*n* = 157	*n* = 136		*n* = 140	*n* = 130	
18:2*n-6*	10.8 (10.6,11.1)	11.1 (10.9,11.3)	0.112	10.8 (10.5,11.0)	11.1 (10.9,11.4)	0.02	10.8 (10.6,11.1)	11.1 (10.8,11.3)	0.171
18:3*n-6*	-	-	-	-	-	-	-	-	-
20:3*n-6*	1.7 (1.7,1.8)	2.0 (1.9,2.1)	4.9×10^{-16}	1.71 (1.7,1.8)	2.1 (2.0,2.1)	2.9×10^{-19}	1.7 (1.7,1.8)	2.05 (1.9,2.1)	6.0×10^{-15}
20:4*n-6*	16.9 (16.6,17.3)	16.3 (16.0,16.7)	0.019	16.9 (16.6,17.3)	16.1 (15.8,16.5)	8.00×10^{-4}	16.9 (16.6,17.3)	16.3 (15.9,16.6)	0.006
22:4*n-6*	2.1 (2.7,3.3)	2.8 (2.7,2.9)	0.441	2.9 (2.7,3.2)	2.9 (2.7,2.9)	0.319	2.9 (2.70,3.3)	2.8 (2.7,2.9)	0.356
18:3*n-3*	0.2 (0.2,0.2)	0.2 (0.2,0.2)	0.472	0.2 (0.2,0.2)	0.2 (0.2,0.2)	0.057	0.2 (0.2,0.2)	0.2 (0.2,0.2)	0.29
20:5*n-3*	1.3 (1.3,1.4)	1.2 (1.2,1.3)	0.034	1.4 (1.3,1.4)	1.2 (1.2,1.3)	0.038	1.3 (1.2,1.4)	1.2 (1.2,1.3)	0.096
22:5*n-3*	3.3 (3.2,3.4)	3.2 (3.1,3.2)	0.032	3.3 (3.2,3.4)	3.1 (3.1,3.2)	0.024	3.3 (3.2,3.4)	3.2 (3.1,3.2)	0.053
22:6*n-3*	6.5 (6.3,6.8)	6.2 (5.9,6.4)	0.041	6.6 (6.4,6.9)	6.1 (5.9,6.4)	0.008	6.5 (6.3,6.8)	6.2 (5.9,6.4)	0.054
Desaturase activity									
D5D[b]	9.9 (9.7,10.3)	8.3 (7.9,8.5)	2.7×10^{-16}	10.0 (9.8,10.3)	8.0 (7.7,8.3)	1.6×10^{-21}	9.9 (9.7,10.3)	8.2 (7.9,8.5)	1.9×10^{-16}

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids and mean surrogate estimates of D5D activity based on ratio of *n-6* product:substrate (95% CI).

[a]Genotype association with fatty acids and desaturase activity was tested respectively by multivariate and univariate analysis of variance based on a dominant model. *P*-values adjusted for BMI, age, gender and ethnicity.

[b]Activity estimated by 20:4*n-6*:20:3*n-6* ratio.

In view of observed associations with plasma LC-PUFA proportions, we examined associations with concentrations of plasma TC, LDL-C, HDL-C, TAG and TC:HDL-C ratio, but none were significant ($P > 0.05$).

5.8.4 Haplotype reconstruction

Any one of a set of SNPs in LD associated with a phenotype is potentially functional and could be the origin of associations seen with the others. Alternatively, all SNPs could be in LD with an unknown functional site. A third possibility exists, whereby an unknown variant on the same haplotype in LD with a functional site could be responsible for associations seen with analysed SNPs. Thus, if haplotypes show stronger associations than SNPs independently, an unknown variant(s) could be responsible for the observed association.

Statistical reconstruction of haplotypes in unrelated individuals, for whom phase is unknown, is possible when SNPs are in strong LD. As only rs174537 has been genotyped in NCBI HapMap trios (http://hapmap.ncbi.nlm.nih.gov/), we could not use the NCBI Haploview program to establish pairwise LD between our SNPs. Instead we used the online program CubeX (*see* Methods Chapter) based on diplotypes available for 282 MARINA subjects. Strong LD was confirmed; the pairwise squared correlations r^2 ranged from 0.83 to 0.99 and Lewontin's D' from 0.98 to 1.00 (**Figure 5.1**).

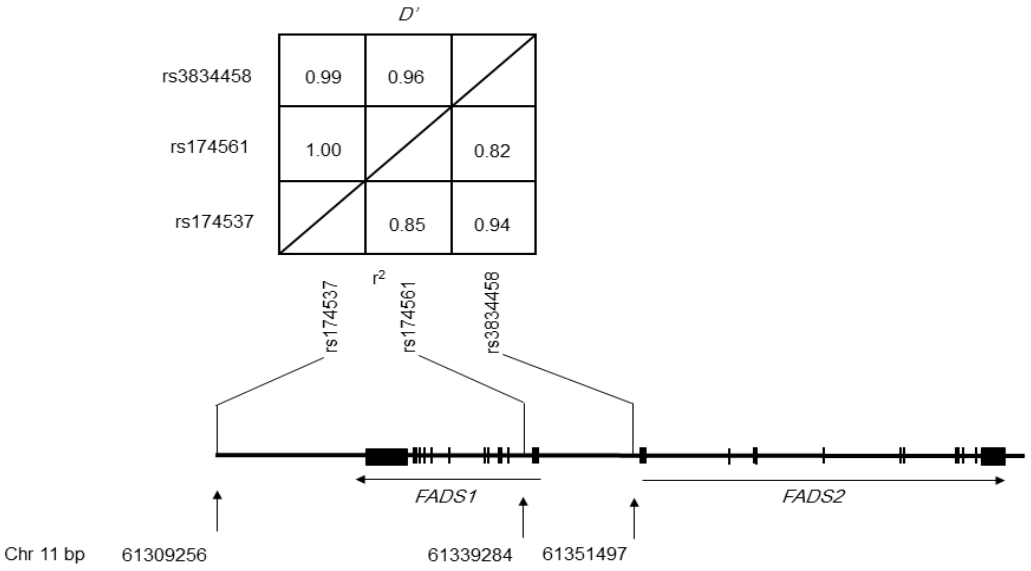


Figure 5.1 Structure of the *FADS1 FADS2* gene cluster, its location on chromosome 11 and pairwise LD D' and r^2 plots of the 3 studied SNPs.

Reconstruction of haplotypes of the three SNPs (rs174537- rs174561- rs3834458) was based on participants for whom all genotypes were available, to avoid errors resulting from missing data. In these, fatty acid composition in plasma and erythrocyte phosphoglycerides was available for respectively 250 and 244 and estimated desaturase activities for 254 subjects. Seven of the eight possible haplotypes were represented, with frequencies ranging from 0.2% to 69.3% (**Table 5.6**). The most common (haplotype 1) carried the major alleles G-T-T at all loci (frequency 69.3%). The next

most frequent (haplotype 2) carried the minor alleles T-C-del at all loci (frequency 25.8%).

Table 5.6 Characteristics of 3-locus haplotypes.

Haplotype number	*FADS* SNP			Haplotype frequency %
	rs174537	rs174561	rs3834458	
Haplotype 1 (Reference)	1	1	1	69.3
Haplotype 2	2	2	2	25.8
Haplotype 3	2	1	2	3.3
Haplotype 4	2	1	1	0.6
Haplotype 5	2	2	1	0.6
Haplotype 6	1	2	1	0.2
Haplotype 7	1	1	2	0.2
Haplotype 8	1	2	2	-

Estimated reconstructed haplotype frequencies under linkage disequilibrium based on subjects with no missing genotype data. 1=major allele; 2=minor allele.

5.8.5 Haplotype associations with plasma and erythrocyte variables at baseline

The significance of haplotype associations with plasma and erythrocyte phenotypes and proportions of variance explained by the three most frequent haplotypes, accounting for 98.4% of the total is shown in **Table 5.7**. Plasma LC-PUFAs associated with independent SNP genotypes (**Table 5.4**) were also associated with haplotype, with similar levels of significance. Variability in fatty acid proportions explained ranged from 26.06% for AA to 2.88% for ALA. The significance of the association between haplotype and estimated D5D activity (P= 2.19 x 10^{-17}) was similar to that for the single SNPs (5.84 x $10^{-19} \leq P \leq$ 4.5 x 10^{-18}) (**Table 5.4**). However, the significance of haplotype association with estimated D6D activity (P= 3.39 x 10^{-28}) was far greater than those with the single SNPs (6.05 x $10^{-8} \leq P \leq$ 4.20 x 10^{-7}).

Among LC-PUFA in erythrocyte phosphoglycerides, DGLA showed by far the strongest association with haplotype, accounting for a much larger proportion of the variance than in plasma (34.6% compared to 10.9%). The significance of association between haplotype and D5D activity estimated in erythrocytes (P= 2.01 x 10^{-19}) was similar to that for the single SNPs (1.60 x $10^{-21} \leq P \leq$ 2.68 x 10^{-16}). No haplotype associations with concentrations of plasma lipids were significant (P> 0.05).

Owing to the relatively small number of subjects with complete genotype and phenotype data (n= 244-256), only the two haplotypes with an expected frequency > 5% were modelled to determine haplotype effects. **Table 5.8** shows the effect on phenotypes of a single copy of haplotype 2 compared to reference haplotype 1. The

most significant effects of haplotype 2 carriage were seen in increased plasma proportions of DGLA and decreased GLA, AA, DPA and DHA. Carriage of haplotype 2 significantly decreased plasma estimates of D5D and D6D activities compared to haplotype 1. In erythrocytes, only the increase in DGLA and the decrease in D5D activity with respect to haplotype 1 were of comparable significance.

Table 5.7 Association of *FADS1-FADS2* 3-SNP haplotypes with phenotypes at baseline and % variance explained.

	Plasma		Erythrocytes	
Phenotype	*P* haplotype association[a]	% variance[b]	*P* haplotype association	% variance
Fatty acids				
18:2*n-6*	0.050	22.4	0.032	11.5
18:3*n-6*	9.80×10^{-9}	17.6	-	-
20:3*n-6*	3.13×10^{-5}	10.9	1.81×10^{-21}	34.5
20:4*n-6*	1.38×10^{-16}	26.1	0.007	5.2
22:4*n-6*	4.02×10^{-4}	7.4	0.382	1.4
18:3*n-3*	0.050	2.9	0.535	2.2
20:5*n-3*	2.34×10^{-4}	8.7	0.023	7.5
22:5*n-3*	7.41×10^{-7}	15.6	0.036	5.7
22:6*n-3*	1.91×10^{-4}	11.9	0.029	6.4
Desaturase activity				
D5D[c]	2.19×10^{-17}	26.2	2.01×10^{-19}	28.9
D6D[d]	3.39×10^{-28}	17.2	-	-

[a]*P*-values adjusted for BMI, age, gender and ethnicity.

[b]Variance explained by most frequent haplotypes 111, 222 and 212 accounting for 98.4% of total.

[c]Activity estimated by 20:4*n-6*:20:3*n-6* ratio.

[d]Activity estimated by 18:3*n-6*:18:2*n-6* ratio. No data available for erythrocytes

Table 5.8 Difference in proportion of plasma and erythrocyte fatty acids and indices of desaturase activity per copy of minor allele haplotype compared to reference at baseline.

	Plasma			Erythrocytes		
	Haplotype 1 reference	Haplotype 2		Haplotype 1 reference	Haplotype 2	
rs174537-rs174561-rs3834458 alleles	1-1-1	2-2-2		1-1-1	2-2-2	
Haplotype frequency %	68.4	27.2		68.4	27.2	
	Intercept	Difference (95% CI)	*P*-value[c]	Intercept	Difference (95% CI)	*P*-value
18:2*n-6*	20.700	0.627 (-0.080 - 1.333)	0.08	7.544	0.304 (0.033 - 0.575)	0.03
18:3*n-6*	0.108	-0.090 (-0.122 - -0.057)	$<1.0 \times 10^{-6}$		-	-
20:3*n-6*	0.489	0.136 (0.076 - 0.196)	8.0×10^{-6}	0.973	0.317 (0.264 - 0.370)	$<1.0 \times 10^{-6}$
20:4*n-6*	3.250	-1.138 (-1.425 - -0.851)	$<1.0 \times 10^{-6}$	7.495	-0.651 (-1.059 - -0.244)	0.002
22:4*n-6*	0.415	-0.036 (-0.064 - -0.009)	0.01	1.442	-0.173 (-0.618 - 0.272)	0.45
18:3*n-3*	0.347	0.045 (0.008 - 0.083)	0.02	0.161	0.011 (-0.026 - 0.048)	0.57
20:5*n-3*	0.144	-0.178 (-0.307 - -0.050)	0.007	0.325	-0.100 (-0.197 - -0.003)	0.04
22:5*n-3*	0.287	-0.055 (-0.078 - -0.032)	2.0×10^{-6}	1.493	-0.128 (-0.240 - -0.016)	0.03
22:6*n-3*	1.129	-0.259 (-0.395 - -0.123)	1.9×10^{-4}	2.960	-0.336 (-0.638 - -0.034)	0.03
20:4*n-6*:20:3*n-6*	2.925	-1.003 (-1.242 - -0.765)	$<1.0 \times 10^{-6}$	4.052	-1.469 (-1.758 - -1.180)	$<1.0 \times 10^{-6}$
18:3*n-6*:18:2*n-6*	0.000	-0.003 (-0.005 - -0.002)	5×10^{-6}	NA	NA	NA-

Haplotype effects based on subjects with no missing genotype data: plasma fatty acids *n* = 250, RBC fatty acids *n* = 244; D5D and D6D desaturase activity *n* = 254. Mean proportion of fatty acid (%) estimated for reference haplotype 1 (major allele 1 at each locus). Mean difference (95% CI) in proportion of fatty acid (%) estimated for haplotype 2 (minor allele 2 at each locus) and haplotype 1. *P*-values adjusted for BMI, age, gender and ethnicity.

5.8.6 Changes in proportions of LC-PUFAs and desaturase activities in plasma and erythrocytes after EPA+DHA treatment

In the second part of the study, we investigated the effects on plasma and erythrocyte phenotypes of dietary supplementation with EPA+DHA (1.51:1) for 6 months. We first assessed changes in proportions of LC-PUFAs, estimated desaturase activities and plasma lipids. We then determined whether genetic associations seen at baseline were modulated by the treatment.

As shown in **Table 5.9**, there were significant changes after treatment in the proportions of all plasma LC-PUFAs except adrenic acid, which is rapidly taken up from plasma by erythrocytes. The proportions of all other *n*-6 LC-PUFAs were significantly decreased and all *n*-3 LC-PUFAs were significantly increased. The fall in *n-6* LC-PUFA is probably due to two factors: decreased conversion from LA caused by inhibition of D6D and secondly competition by *n*-3 LC-PUFA at the level of acyl transferase where the fatty acids are incorporated into the lipids. There was a significant increase in D5D and a significant decrease in D6D activity after supplementation. There were no significant effects of haplotype on changes in the concentrations of plasma lipids (TC, HDL-C, TAG) after treatment.

There were significant changes in proportions of all LC-PUFAs except ALA in erythrocyte phosphoglycerides after treatment. The proportions of all other *n*-3 LC-PUFAs significantly increased and *n*-6 LC-PUFAs significantly decreased. The change

in estimated D5D activity in erythrocytes was barely significant at the $P < 0.05$ level. Erythrocyte data are shown in **Table 5.10.**

Table 5.9 Proportion of fatty acids and desaturase indices in plasma stratified by treatment group after 6 months randomized treatment.

	Placebo	0.45 g/day	0.9 g/day	1.8 g/day	*P*
	n = 71	*n* = 82	*n* = 81	*n* = 82	
18:2*n-6*	27.57 (26.93,28.20)	27.21 (26.61,27.80)	26.74 (26.14,27.33)	26.28 (25.68,26.88)	0.016
18:3*n-6*	0.47 (0.44,0.49)	0.42 (0.39,0.44)	0.39 (0.36,0.42)	0.32 (0.29,0.35)	8.77×10^{-9}
20:3*n-6*	1.62 (1.57,1.68)	1.47 (1.42,1.52)	1.41 (1.36,1.46)	1.14 (1.09,1.19)	3.26×10^{-26}
20:4*n-6*	6.80 (6.60,6.99)	6.55 (6.37,6.73)	6.45 (6.27,6.63)	6.04 (5.86,6.22)	1.80×10^{-5}
22:4*n-6*	0.60 (0.57,0.62)	0.60 (0.57,0.62)	0.61 (0.59,0.64)	0.63 (0.61,0.65)	0.215
18:3*n-3*	0.60 (0.57,0.64)	0.66 (0.62,0.70)	0.63 (0.59,0.67)	0.68 (0.64,0.72)	0.008
20:5*n-3*	0.94 (0.74,1.13)	1.86 (1.68,2.04)	2.37 (2.19,2.55)	3.88 (3.70,4.06)	3.86×10^{-65}
22:5*n-3*	0.62 (0.59,0.65)	0.74 (0.71,0.76)	0.79 (0.76,0.81)	0.93 (0.90,0.95)	1.47×10^{-37}
22:6*n-3*	2.27 (2.16,2.39)	2.86 (2.75,2.97)	3.39 (3.28,3.50)	4.04 (3.93,4.15)	4.16×10^{-52}
Desaturase activity					
18:3*n-6*:18:2*n-6*	4.37 (4.06,4.68)	4.63 (4.31,4.94)	4.93 (4.58,5.28)	5.45 (5.10,5.79)	4.00×10^{-9}
18:3*n-6*:18:2*n-6*	0.018 (0.016,0.019)	0.016 (0.015,0.017)	0.015 (0.014,0.016)	0.013 (0.012,0.014)	7.56×10^{-6}

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids and mean surrogate estimates of D5D and D6D activities based on ratio of *n-6* product:substrate (95% CI), all adjusted for baseline values. Measurements made after 6 months on placebo or EPA+DHA supplements are shown for each randomized treatment group.Significance of difference in change in proportions of fatty acids with respect to baseline between treatment groups was tested by multivariate analysis of variance. Significance of differences in desaturase activities adjusted for baseline values was tested by univariate analysis of variance. All *P*-values adjusted for BMI, age, gender and ethnicity.

Table 5.10 Proportion of fatty acids and desaturase indices in erythrocytes stratified by treatment group after 6 months randomized treatment.

	Placebo	0.45 g/day	0.9 g/day	1.8 g/day	P^1
	n = 71	*n* = 82	*n* = 81	*n* = 82	
18:2*n-6*	11.04 (10.79,11.29)	10.79 (10.56,11.03)	10.76 (10.53,11.00)	10.15 (9.91,10.38)	3.32×10^{-5}
20:3*n-6*	1.88 (1.82,1.94)	1.75 (1.70,1.81)	1.66 (1.61,1.72)	1.48 (1.42,1.53)	9.05×10^{-15}
20:4*n-6*	16.58 (16.23,16.92)	15.66 (15.33,15.98)	15.26 (14.94,15.59)	13.94 (13.61,14.26)	1.56×10^{-12}
22:4*n-6*	3.14 (2.93,3.34)	2.43 (2.23,2.62)	2.28 (2.09,2.48)	1.91 (1.72,2.11)	5.75×10^{-6}
18:3*n-3*	0.17 (0.14,0.19)	0.17 (0.15,0.19)	0.16 (0.14,0.18)	0.20 (0.17,0.22)	0.408
20:5*n-3*	1.18 (1.02,1.33)	1.97 (1.83,2.12)	2.48 (2.33,2.62)	3.65 (3.51,3.79)	1.87×10^{-66}
22:5*n-3*	3.15 (3.05,3.26)	3.52 (3.43,3.61)	3.81 (3.71,3.91)	4.18 (4.06,4.29)	1.06×10^{-28}
22:6*n-3*	6.21 (5.96,6.46)	7.03 (6.80,7.27)	7.83 (7.59,8.06)	8.32 (8.09,8.56)	8.34×10^{-21}
Desaturase activity					
D5D[2]	9.20 (8.74,9.65)	9.14 (8.71,9.57)	9.67 (9.24,10.10)	9.73 (9.30,10.15)	0.046

Data show mean (95% CI) for each LC-PUFA as a % of total fatty acids and mean surrogate estimate of D5D activity based on ratio of *n-6*product:substrate (95% CI), all adjusted for baseline values. Measurements made after 6 mo on placebo or EPA+DHA supplements are shown for each randomized treatment group.

[1]Significance of difference in change in proportions of fatty acids with respect to baseline between treatment groups was tested by multivariate analysis of variance. Significance of difference in desaturase activity adjusted for baseline value was tested by univariate analysis of variance. All *P*-values adjusted for BMI, age, gender and ethnicity.

[2]Activity estimated by 20:4*n-6*:20:3*n-6* ratio.

5.8.7 SNP associations with plasma and erythrocyte variables after dietary intervention

There were no significant differences in SNP genotype frequencies between the four treatment groups: rs174537 χ^2 = 6.79, 6 df, *P* = 0.34; rs174561 χ^2 = 9.62, 6 df, *P* = 0.14; rs3834458 χ^2 = 9.00, 6 df, *P* = 0.17. We found no significant differences in LC-PUFA proportions between common homozygotes and carriers of the minor allele of any of the three SNPs in any treatment group (*P*> 0.05). There were also no effects on plasma lipids dependent on genotype and dose. However, there were significant effects of treatment on estimations of desaturase activity when stratified by genotype. Carriers of the rs174537 minor T-allele had significantly lower D5D activity than GG subjects in the placebo group, but with increasing dosage, activity increased significantly in T-carriers (*P* = 3.2 x 10^{-10} in plasma and *P* = 4.3 x 10^{-4} in erythrocytes), but not in GG homozygotes (*P* = 0.11 in plasma, *P* = 0.76 in erythrocytes) as shown in **Figure 5.2**. Interaction between rs174537 genotype and intake as a determinant of D5D activity was significant at the *P*< 0.05 level (*P* = 0.05 for plasma and *P* = 0.02 for erythrocyte estimates) after adjustment for age, BMI, ethnicity, gender and D5D activity at baseline but before correction for multiple comparisons. After correction, interaction remained significant for the plasma estimate (*P* = 0.05) but not for the erythrocyte measure (*P* = 0.20). Interaction between treatment and rs174561 genotype was not significant (*P*> 0.05). Interaction with rs3834458 genotype was significant for the determination in plasma before correction for multiple testing (*P* = 0.05), but not after correction (*P* =

0.15). There was no significant interaction between any of the SNP genotypes and treatment in determining D6D activity.

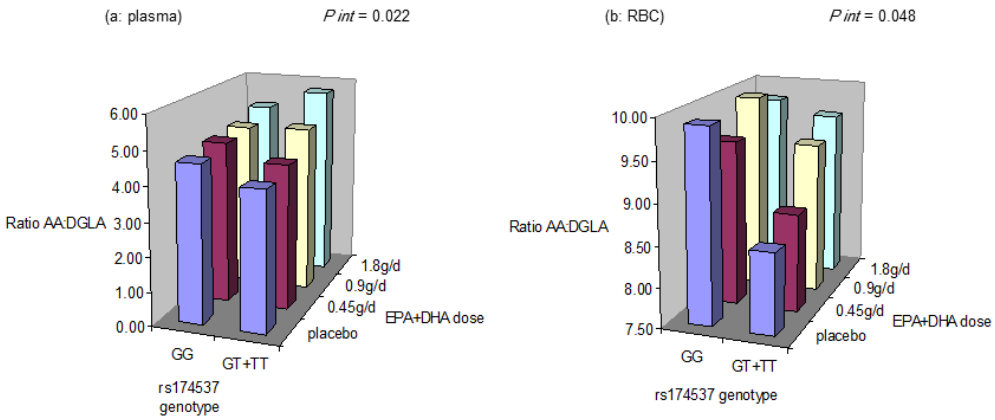


Figure 5.2 Effect of increasing EPA+DHA dose on D5D activity in carriers of the minor allele (T).

5.8.8 Spearman's correlation between erythrocyte phospholipids, total plasma fatty acids and dietary fatty acids

Spearman's correlation between the individual fatty acid of the erythrocyte phospholipids and plasma total fatty acids after 6 months intervention and those of the FFQ are respectively illustrated in **Table 5.11**. The correlation between available fatty acids from the FFQ and those of erythrocyte phospholipids and total plasma revealed a significant correlation for PUFA (RBC: ρ=0.128, *P*=0.02; Plasma: ρ=0.208, *P*<0.001), EPA (RBC: ρ=0.196, *P*=0.001; Plasma: ρ=0.162, *P*=0.005), DHA (RBC: ρ=0.127, *P*=0.02; Plasma: ρ=0.199, *P*<0.001) and the plasma SFA (ρ=0.125, *P*=0.03), but not those of erythrocyte phospholipids. ALA (RBC: ρ=0.075, *P*=0.186; Plasma: ρ=0.09, *P*=0.116) showed a very weak correlation for the two biomarkers and corresponding ones from the FFQ. While MUFA (RBC: ρ=-0.005, *P*=0.0.934; Plasma: ρ= -0.066, *P*=0.252) and LA (RBC: ρ=-0.025, *P*=0.62; Plasma: ρ=-0.027, *P*=0.637) showed a very weak negative correlation.

Table 5.11 Spearman's correlations between the proportions of different fatty acids in erythrocyte lipids and total plasma fatty acids with those in the diet estimated from food frequency questionnaire

	Erythrocytes		Plasma	
	P	*P*-value	ρ	*P*-value
SFA	0.025	0.658	0.125	0.03
MUFA	-0.005	0.934	-0.066	0.252
PUFA	0.128	0.023	0.208	<0.001
LA	-0.0257	0.652	-0.0272	0.637
ALA	0.075	0.186	0.09	0.116
EPA	0.196	0.001	0.162	0.005
DHA	0.127	0.025	0.199	<0.001

5.8.9 Possible factors influencing Carotid intima-medial thickness (CIMT) assessed by high resolution ultrasound at baseline

Baseline carotid diameter, intima-media thickness (IMT) and presence of atherosclerotic plaque in participants as detected by high resolution ultrasound stratified by gender are illustrated in **Table 5.12**. Left and right minimal carotid diameters were higher in males than females. The same applies to left and right maximal carotid diameters. Left and right anterior IMT and posterior IMT were also higher in males than females.

Further analysis shows CIMT thickness stratified by erythrocytes phospholipids omega-3 index (EPA+DHA) tertiles (**Table 5.13**). There was no significant relationship between the omega-3 index and CIMT adjusted for age, gender, carotid diameted, systolic BP and TC:HDL-C.

Table 5.12 Carotid diameter, intima-media thickness (IMT) and presence of atherosclerotic plaque in participants as detected by high resolution ultrasound.

	Left		Right	
	Males	Females	Males	Females
	n=118	*n*=193	*n*=118	*n*=193
Minimal carotid diameter (mm)	7.03 ± 0.64	6.38 ± 0.59	7.21 ± 0.74	6.57 ± 0.65
Maximal carotid diameter (mm)	7.51 ± 0.61	6.81 ± 0.71	7.69 ± 0.79	7.03 ± 0.63
Anterior IMT (mm)	0.67 ± 0.14	0.64 ± 0.13	0.66 ± 0.15	0.64 ± 0.15
Posterior IMT (mm)	0.64 ± 0.17	0.60 ± 0.14	0.61 ± 0.14	0.57 ± 0.11
Carotid plaque (%)[1]	10.20%	7.20%	7.60%	8.80%

[1]% of total number.

Table 5.13 CIMT thickness stratified by tertiles of erythrocyte phospholipids omega-3 index (EPA+DHA).

	Omega-3 index			
CIMT	Low	Medium	High	*P* value
Left anterior	0.66 (0.64,0.68)	0.65 (0.63,0.67)	0.63 (0.61,0.65)	0.12
Left posterior	0.62 (0.60,0.65)	0.62 (0.60,0.65)	0.60 (0.57,0.63)	0.231
Right anterior	0.64 (0.61,0.67)	0.66 (0.64,0.69)	0.64 (0.62,0.67)	0.868
Right posterior	0.59 (0.57,0.61)	0.59 (0.57,0.61)	0.59 (0.57,0.61)	0.964

Values are mean (95% CI).

Statistical significance is for linear trend.

Analysis adjusted for age, gender, SBP, TC:HDL-C, carotid diameter.

5.9 Discussion

In this sample of healthy subjects, the genotypes of three SNPs in the *FADS1-FADS2* gene cluster were strongly associated with proportions of LC-PUFAs and desaturase activities estimated in plasma and erythrocytes. Minor allele carriage associated with decreased activity of D5D (*FADS1*) and D6D (*FADS2*) was reflected in increased proportions of substrates and decreased products in *n*-6 and *n*-3 LC-PUFA synthetic pathways. SNPs and haplotype associations with LC-PUFA proportions and D5D activity of similar significance suggested that the analysed SNPs were in LD with a potential functional site. However, D6D activity was much more strongly associated with haplotype than with single SNP, suggesting an unknown variant on the same haplotype might be influential. We have shown that increasing dosage of EPA and DHA in a randomized controlled trial reduced *n*-6 and increased *n*-3 LC-PUFA proportions, and D5D activity. SNP genotypes did not interact with treatment in determination of LC-PUFA proportions, but interaction was a significant determinant of D5D activity.

The composition of fatty acids in tissues reflects the dietary fat composition, but individual differences reflect genetic control of metabolic efficiency. The D5D and D6D genes, *FADS1* and *FADS2*, are important regulators of LC-PUFA synthesis, evidenced by the extremely high genetically explained variance of AA (Tanaka *et al*. 2009). As in other investigations (Zietemann *et al*. 2010; Bokor *et al*. 2010), separate surrogate estimates of D5D and D6D activities was used based on ratios of LC-PUFA

product:substrate in the *n*-6 pathway to assess the influence of *FADS* genetic variants. This study confirmed highly significant association of *FADS* SNP minor alleles with a reduction in desaturase activities estimated in plasma and erythrocytes. This may indicate a decline in gene transcription and/or enzyme conversion rates in carriers and would result in the increased substrate and decreased product proportions generally observed. At baseline, we found several highly significant SNP minor allele associations with increased proportions of substrates and decreased proportions of products in plasma and a strong association with increased DGLA in erythrocytes. Previous studies have shown association of several SNPs in this region with LC-PUFAs in plasma (Martinelli *et al*. 2008; Schaeffer *et al.* 2006) and erythrocyte membranes (Tanaka *et al*. 2009; Xie *et al*. 2008) although most did not find associations with proportions of DHA, for which sources are thought to be mainly nutritional (Schaeffer *et al*. 2006). The higher D5D activity in erythrocytes than in plasma may have reflected a preferential incorporation of AA into erythrocyte membrane phosphoglycerides. Recent GWA studies have also identified several genetic loci in the *FADS* gene cluster that are associated with blood lipid levels (Aulchenko *et al*. 2009; Sabatti *et al*. 2009; Chasman *et al*. 2009; Kathiresan *et al*. 2009). However, we were unable to replicate previously reported associations with plasma lipids, particularly LDL-C (Lu *et al*. 2010; Nakayama *et al*. 2010; Hellstrand *et al*. 2012). This most likely relates to the fact that lipids are distal phenotypes, which are influenced by many genes and environmental factors in addition to desaturase activity. Moreover, this study sample lacked the power to detect significant *FADS* genotype associations at baseline. As expression is highest in

the liver (Cho *et al*. 1999a; Cho *et al*. 1999b), a contribution of *FADS1* and *FADS2* genetic variation to plasma cholesterol metabolism seems likely.

The *FADS1* and *FADS2* genes have inverse orientation as a cluster on chromosome 11, with exon 1 of both genes separated by an 11 kb region (Lattka *et al*. 2010). The proximity of the promoters suggests that their transcription may be co-ordinately controlled by common regulatory sequences (Nakamura *et al*. 2004). Common genetic variants at the *FADS1-FADS2* locus are in a strong LD block spanning *FADS1* and the intergenic region (Martinelli *et al*. 2008; Tanaka *et al*. 2009; Schaeffer *et al*. 2006) so that any functional polymorphisms within the block could influence expression of both desaturases. However, our haplotype analysis suggests that control of D5D and D6D activity may have different genetic origins. SNP rs3834458 sited 5' to *FADS2* is a good candidate, but an effect on promoter activity has not been established (Lattka *et al*. 2010). Proportions of fatty acids and D5D activity in plasma and erythrocytes associated with single SNPs at baseline were also associated with the minor allele haplotype with similar levels of significance. The analysed SNPs seem, therefore, to be LD markers of a site influencing D5D activity. However, the significance of haplotype association with D6D activity was substantially greater than that of single SNPs, suggesting that unknown functional SNPs or possibly more than one causal variant on the haplotype influences D6D. Further support comes from a recent GWA study of gene expression, in which rs174546, in LD with our analysed SNP rs174537 (r^2=0.99) was

associated with *FADS1* (P=1.6 × 10^{-6}) but not *FADS2* (P=0.07) expression in lymphoblastoid cells (Dixon *et al*. 2007).

As enzymes of the synthetic pathways show a higher affinity for *n*-3 than *n*-6 PUFA, the effect of treatment with EPA+DHA was to increase competition from derivatives of the *n*-3 LC-PUFA supplements and decrease proportions of the *n*-6 series. The significant increase in D5D activity after treatment reflected a greater reduction in the product, AA than in the substrate, DGLA. Modulations of the activities of D5D and D6D by intakes of EPA+DHA have been detected in previous controlled intervention studies (Zhou & Nilsson, 2001; Vessby *et al*. 2002). Interaction between intake of *n*-3 PUFA or fatty fish and *FADS* genotypes have been established in larger studies by some (Hellstrand *et al*. 2012) but not others (Lemaitre *et al*. 2011; Lu *et al*. 2010; Moltó-Puigmartí *et al*. 2010). Interaction between genotype and treatment as a determinant of LC-PUFA proportions was not significant in plasma or erythrocytes in the present study, most likely owing to insufficient power. For example, to demonstrate a significant difference in proportion of plasma AA with respect to rs174537 genotype across treatments based on a dominant model, a total sample size of 941 would be required for alpha = 0.05 and a power of 0.95. To demonstrate a significant difference based on an additive model, a total sample size of 1240 would be required. However, this study was unable to find that interaction was a significant determinant of D5D activity estimated in plasma, which increased significantly with dose in variant allele carriers with virtually no change in the common homozygotes. The effect in

erythrocytes appeared even more pronounced, but was not significant after correction for multiple comparisons. D6D activity decreased significantly with treatment, but not after stratification by genotype. LC-PUFAs have previously been shown to down-regulate D6D and increase D5D activity in controlled dietary studies (Vessby *et al*. 2001).

5.10 Limitations and conclusion

In summary, this study was able to demonstrate that the consumption of as low as 0.45g of preformed EPA+DHA, which is equivalent of one portion of oily fish, was powerful in increasing proportions of these two fatty acids and their metabolites in erythrocyte phospholipids and plasma. Differences in levels of fatty acids between the two biomarkers indicate higher affinity of the erythrocyte cell membrane over the plasma. In addition, the plasma tends to reflect short-term dietary intake, while the erythrocyte represent medium-term intake and tightly regulated.

The greatest strength of this study was the strict control of the intakes of EPA and DHA, shown to be correlated with plasma proportions. Long-term compliance was established by measures in erythrocytes. Although adjustments of probabilities for multiple testing were done by using multivariate analysis of variance, the best insurance that these results are not due to chance lies in replication in an independent sample. However, most associations between LC-PUFA proportions and *FADS* variants at baseline were

highly significant after correction for multiple comparisons and agree with findings in larger studies.

The main limitation was the relatively small sample of subjects for genetic analysis (n = 310), which reduced the power to detect some significant genotype associations with phenotypes and interactions with diet. Location of the three SNPs within a strong LD block on the one hand makes identification of the cause of the observed associations difficult, but on the other, high correlation between genotypes enabled haplotype reconstruction and analysis. Like other investigators, this study used LC-PUFA ratios as surrogate desaturase activities, because direct measures are not possible in population studies.

This study confirmed that *FADS* polymorphisms are an important regulator of LC-PUFA synthesis through high genetically explained variance of several fatty acids. Haplotypes carrying three SNP minor alleles were associated with lower indices of D5D and D6D activities, suggesting that any could be in linkage disequilibrium with a functional SNP. However, this study has raised the possibility that another variant on the same haplotype might have more influence on indices of D6D activity than the studied SNPs. In this relatively small sample, it was demonstrated significant interaction between dietary *n*-3 LC-PUFA intake and rs174537 genotype as a determinant of indices of D5D activity, with potential effects on the composition of LC-PUFA depots and implications for health.

Chapter 6: Final discussion and conclusions

This thesis set out to evaluate the use of biomarkers of fatty acid intake by testing how they performed in large randomized controlled trials of modification of fatty acid intake. The conclusion from the CRESSIDA study is that the proportion of SFA in erythrocyte lipids did not change when SFA intake was decreased and only minor changes occurred in SFA were observed in plasma lipids. The RISCK study (Jebb *et al*. 2010), which carefully controlled SFA intake by lowering the intake of SFA by up to 10% through replacing them with either carbohydrates or MUFA, found plasma phospholipids fatty acid composition to be a poor indicator of dietary intake of SFA (**Table 6.1**). Further analyses by the author on a subset of plasma samples from RISCK study also failed to show any differences in the proportions of SFA between groups fed diets high in MUFA or carbohydrate compared to the control diet (**Table 6.2**). In contrast, the blood biomarkers were very sensitive to change in PUFA intake and particularly to increased intakes of LC *n*-3 PUFA. Other trials have shown that fatty acids not synthesised *de novo* in the body such as branched fatty acids (C15:0 and C17:0) and TFA are also reflected in these and other lipid biomarkers (Sanders *et al.* 2003). The data from the TwinsUK study showed that PUFA and TFA in adipose tissue and plasma both correlated with the proportion in diet and are, therefore, good biomarkers of dietary intake. The reason for the lack of sensitivity of the biomarkers to SFA and MUFA is likely to be that homeostatic mechanisms keep the proportions of palmitic and oleic acids relatively constant.

Table 6.1 Intakes of SFA and MUFA and changes in plasma phospholipid SFA and MUFA in the RISCK study.

Diet	SFA *n*=78	HM/HGI *n*=106	HM/LGI *n*=103	LF/HGI *n*=102	LF/LGI *n*=109
SFA % energy					
Baseline	17.0 ± 3.1	17.0 ± 3.1	17.0 ± 3.1	17.0 ± 3.1	17.0 ± 3.1
Follow-up	16.9 ± 3.3	10.1 ± 1.3	9.4 ± 2.6	9.4 ± 2.9	8.4 ± 2.6
MUFA % energy					
Baseline	12.0 ± 2.2	12.0 ± 2.2	12.0 ± 2.2	12.0 ± 2.2	12.0 ± 2.2
Follow-up	12.2 ± 2.8	16.9 ± 4.9	16.3 ± 4.3	10.4 ± 3.3	10.0 ± 3.1
Plasma phospholipids %SFA					
Baseline	44.3 (43.4,45.4)	44.7 (43.5,45.9)	45.0 (43.7,45.7)	44.6 (43.2,45.6)	44.6(43.2,45.6)
Follow-up	44.3 (43.3,45.5)	44.4 (43.2,45.5)	44.4 (43.3,45.5)	44.7 (43.7,45.6)	44.7(43.5,45.8)
%MUFA					
Baseline	11.3 (10.4,12.5)	11.6 (10.4,12.5)	11.4 (10.6,12.0)	11.5 (10.7,12.5)	11.3(10.6,12.6)
Follow-up	11.2 (10.5,12.5)	12.4 (11.0,13.6)	12.3 (11.5,13.1)	11.8 (10.7,12.6)	12.0(10.9,12.8)

SFA= control diet, HM/HGI high MUFA low glycemic index diet, HM/LGI low glycemic index diet, LF/HGI low fat high glycemic index diet, LF/LGI low fat low glycemic index diet, Mean values ± SD or median values (interquartile range), Data taken from final report submitted to the Food Standards Agency of the RISCK study (Jebb *et al.* 2010).

Table 6.2 Plasma fatty acid composition (wt%) in the RISCK study comparing the SFA rich diet with low SFA/high MUFA (HM/HGI) diet and low SFA/high carbohydrate (LF/HGI) at the end of the study. Analysis performed by author.

	SFA (*n*= 24)	HM/HGI (*n*= 39)	LF/HGI (*n*= 32)
SFA			
14:0	0.94 ± 0.41	1.00 ± 0.29	0.83 ± 0.38
16:0	17.81 ± 2.8	19.14 ± 1.93	18.40 ± 2.27
18:0	5.81 ± 0.86	6.28 ± 0.71	5.87 ± 0.89
20:0	0.19 ± 0.05	0.20 ± 0.11	0.19 ± 0.04
MUFA			
16:1*n*-7	1.94 ± 0.74	2.13 ± 0.73	1.98 ± 0.68
18:1*n*-9	17.89 ± 3.35	19.96 ± 2.96	18.53 ± 4.43
18:1*n*-7	1.63 ± 0.32	1.76 ± 0.34	1.67 ± 0.41
TFA: 18:1*trans*	0.18 ± 0.09	0.22 ± 0.11	0.20 ± 0.1
***n*-6 PUFA**			
18:2*n*-6	25.19 ±5.1	25.56 ± 4.24	24.93 ± 4.86
18:3*n*-6	0.46 ± 0.19	0.47 ± 0.14	0.43 ± 0.19
20:3*n*-6	1.42 ± 0.39	1.60 ± 0.32	1.44 ± 0.38
20:4*n*-6	6.30 ± 1.74	6.50 ±1.65	5.86 ± 1.85
22:4*n*-6	0.62 ± 0.15	0.59 ± 0.18	0.58 ± 0.18
22:5*n*-6	0.85 ± 0.31	0.79 ± 0.32	0.84 ± 0.27
***n*-3 PUFA**			
18:3*n*-3	0.57 ± 0.22	0.69 ± 0.22	0.62 ± 0.16
20:5*n*-3	1.16 ± 0.68	1.04 ± 0.5	1.15 ± 0.7
22:5*n*-3	0.56 ± 0.11	0.60 ± 0.12	0.58 ± 0.12
22:6*n*-3	2.34 ± 0.63	2.46 ± 0.91	2.39 ± 0.87

Mean values ± SD. No significant differences by multivariate analysis of variance adjusted for age, BMI and gender.

It is interesting to speculate why the proportions of palmitic and stearic acids in biomarkers are so resistant to change. In the case of erythrocytes and other membrane lipids, which are composed primarily of the phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol, there is tighter regulation of the type of fatty acids incorporated into the *sn*-1 and *sn*-2 positions of the phospholipid (Gurr, Harwood & Frayn, 2002). The acyl transferases appear to favour PUFA, especially C20-C22 LC-PUFA, in the *sn*-2 position. Phosphatidylcholine species show selectivity for palmitic acid in the *sn*-1 position rather than stearic acid. However, this does not entirely explain why plasma fatty acid composition is also not a good biomarker of SFA intake. Plasma NEFA and TAG composition tend to reflect a composite mixture of fat from *de novo* lipogenesis and diet. Animals fed on fat free diets synthesise SFA, mainly palmitic acid, but intermediates also accumulate such as lauric and myristic acids. Palmitic acid can react with carnitine to form carnitine esters and be transported for beta-oxidation in the mitochondria. Alternatively, it can be desaturated to 16:1*n*-7 or chain elongated to stearic acid and then desaturated to oleic acid (18:1*n*-9). The same pathways operate in humans as in mammals. However, there is some debate regarding the extent of *de novo* lipogenesis from carbohydrates in humans (McDevitt *et al*. 2001) with probably only 10g/d at most being synthesised from carbohydrate. As dietary fat intakes are typically in the range of 60-100g/d in adults, this suggests that most human body fat is derived from dietary fat. However, this dietary fat can undergo remodelling in the body as it is stored, released into circulation as NEFA, reprocessed in the liver and stored again in adipose tissue. Adipose tissue

itself may also play a role in modifying the composition of the fat. Indeed, adipose tissue does express *SCD-1* (Paton & Ntambi, 2009). The observation that palmitoleic acid accounted for 5-6% in adipose tissue in the Twins study (much more than in plasma lipids), suggests that a substantial portion of palmitic acid is desaturated in adipose tissue. The low proportion of stearic acid and the much greater proportions of oleic acid in adipose tissue than in diet is also consistent with the idea that adipose tissue reprocesses SFA into MUFA.

Controlled feeding studies in mice by our group (Sim, 2011) at King's College London have shown that the proportion of palmitic acid in adipose tissue only increased to 24% compared to when animals were fed carbohydrate where the value was 22%. This would indicate that there are mechanisms that control the proportion of palmitic acid in adipose tissue. Both palmitic acid and oleic acid are good substrates for beta-oxidation although oleic acid is oxidized at a faster rate (Leyton *et al*. 1987). The conversion of palmitic and stearic acids to oleic acid affects the physical properties of fat by lowering the melting point of TAG so that they are in the liquid phase at body temperature. Studies on *SCD-1* knockout transgenic mice have shown that the failure to produce the palmitoleic and oleic acids results in metabolic abnormalities including abnormal secretion of sebum causing alopecia and dry eyes (Guilou *et al*. 2010). In essence, palmitic and oleic acids are the major fat fuels used by mammals for energy production and there are homeostatic mechanism controlling the levels of these fatty acids, which are probably important for maintaining normal physiological functioning.

Further support for the view that plasma fatty acids are unreliable indices of SFA intake is illustrated by comparing vegans whose intakes of SFA are typically less than 6.5% energy with omnivores whose intakes are nearer 12.5% energy (Sanders *et al*. 2009b). Vegans have much higher intakes of LA (about 10% energy) and slightly higher intakes of ALA, but no LC *n*-3 PUFA. **Table 6.3** shows the results of serum fatty acid analysis comparing vegans with omnivores based on samples collected by Lloyd-Wright in our laboratory. Although the proportion of palmitic acid is marginally lower as well as that of palmitoleic acid, the main difference is the very high proportion of LA. The lower levels of EPA and DHA reflect the absence of EPA and DHA from the vegan diet and the relatively high ratio of LA:ALA in the diet. However, it is to be noted that the proportion of AA was slightly higher in the vegans indicating that there were no problems regarding the activities of *FADS-1* and *FADS-2* of converting LA to AA.

The failure to observe any significant relationship between the intake of SFA with the proportion of SFA in adipose tissue of the twins is consistent with the views expressed above that the fatty acid composition of plasma or adipose is not a reliable biomarker of SFA (at least the major dietary SFA: lauric, myristic, palmitic and stearic acids).

Table 6.3 Plasma fatty acid composition in vegans compared with omnivores.

	Vegan (*n*=131)	**Omnivore (*n*=141)**	***P* value**
SFA			
16:0	19.02 ± 2.00	20.82 ± 1.93	<0.001
18:0	6.52 ± 1.08	6.27 ± 0.55	0.047
MUFA			
16:1*n*-7	1.38 ± 0.84	2.25 ± 0.98	<0.001
18:1*n*-9	20.65 ± 3.29	21.75 ± 2.83	0.001
PUFA			
***n*-6**			
18:2*n*-6	36.21 ± 4.91	26.21 ± 3.90	<0.001
20:3*n*-6	1.48 ± 0.42	1.53 ± 0.30	0.106
20:4*n*-6	6.87 ± 1.82	6.24 ± 1.32	0.001
***n*-3**			
18:3*n*-3	0.84 ± 0.52	0.64 ± 0.22	<0.001
20:5*n*-3	0.42 ± 0.26	1.35 ± 0.73	<0.001
22:5*n*-3	0.58 ± 0.39	0.69 ± 0.16	0.012
22:6*n*-3	0.90 ± 0.35	2.74 ± 0.92	<0.001

Mean values ± SD in wt %. Data from Sanders (2009b) and (Z. Lloyd-Wright and TAB Sanders unpublished).

P-value for differences between the two groups obtained from 2 sample *t*-test.

The fatty acid composition of total plasma lipids offers some advantages over total phospholipids or lecithin in that it is more sensitive to changes in ALA intake. The analysis of total plasma lipids is also easier and does not require prior separation of the phospholipids before derivatisation and is, therefore, much more suitable for up-scaling in large cohort studies. Erythrocyte lipids are sensitive indicators of the intake of LC-PUFA, but it is necessary to prepare the lipid extracts within a few days of collection in order to obtain reproducible results. Frozen red blood cells are susceptible to lipid oxidation owing to the presence of iron. The analyses from the MARINA study showed clear dose response relationships between the intake of EPA and DHA and levels in plasma and erythrocytes. There were strong correlations between values for EPA and DHA between biomarkers. Erythrocyte lipids, while more burdensome to prepare lipid extracts, do offer some advantage in that they are less readily changed by diet in the short term and contain higher amounts of C20-C22 LC-PUFA.

Theobald *et al*. (2004) in a cross-over study comparing the effect of 0.85g/d DHA *vs* an olive oil placebo taken for 3 months reported changes in plasma and erythrocyte lipids. She found DHA increased from 5.2% to 8.2% in erythrocytes and plasma DHA increased by 76%. Sanders *et al*. (2006b) in a parallel designed study where subjects consumed 1.5g/d DHA for six weeks reported an increase in erythrocyte DHA from 6.1% to 7.6% and an increase in plasma DHA of 128%, indicating that plasma was more sensitive biomarker. In an RCT in vegans given 0.2g/d DHA or placebo for 3 months, erythrocyte DHA increased from 1.9% to 2.8% in erythrocytes and from 0.9%

to 1.3% in plasma total lipids (Sanders, 2009b; Lloyd-Wright PhD Thesis, 2005). Although the DHA increased, the levels in the vegan still remained much lower than in omnivores probably because of competition from the high intake of LA (Sanders, 2009b). Roshanai & Sanders (1985) in a study in rats demonstrated that high intakes of LA reduced levels of DHA and EPA in tissues even when DHA and EPA were provided preformed in diet.

6.1 Relationship of lipid biomarkers with cardiovascular disease

Khaw *et al*. (2012) using plasma phospholipids as a biomarker of fatty acid intake in the EPIC Norfolk study found higher proportions of SFA and lower proportions of PUFA, particularly LA, to be associated with increased risk of CHD. The relationship seemed strongest for LA. Clark *et al*. (2009) found a low P/S ratio in cholesterol ester to be associated with increased risk in the Whitehall Study. One interpretation could be that the participants at greatest risk had high intakes of SFA but another more plausible explanation is that their intake of LA was low with the consequence that palmitic and oleic acid proportions were increased. Not reported in this thesis, however, is the use of branched chain SFA (mainly C15 and C17) as a biomarker of ruminant fat intake. These fatty acids are products of microbial synthesis and are found in the fat from ruminant animals as well as in fish, where they accumulate from marine plankton. It is of interest that Khaw *et al*. (2012) found a negative association with branched chain fatty acids and risk of CHD because this would suggest that the intake of SFA from butter fat and from lamb and beef was not associated with increased risk. Indeed, it could suggest that the

consumption of butter fat as opposed to margarine was associated with a lower risk of CHD. Another fatty acid that has been found to be associated with dairy fat intake is conjugated linoleic acid (CLA), which occurs in two isoforms. Lloyd-Wright *et al*. (2003) showed the levels of this fatty acid increased from low levels in vegans, intermediate levels vegetarians and higher levels omnivores suggesting it was quite a sensitive indicator of intake. There is currently much interest in the potential beneficial effects of CLA on human health (McCrorie *et al*. 2011).

Harris *et al*. (2007) has proposed that the sum of EPA+DHA in erythrocyte lipids could be used as an index of omega-3 fatty acid status. The work reported in this thesis shows that the index in plasma is strongly correlated with that in erythrocytes. Data from the MESA study (He *et al*. 2008) in 5488 adults aged 45-84 years suggested that omega-3 index was associated with a lower carotid intimal media thickness. The relationship between the omega-3 index and CIMT was investigated in both the MARINA and CRESSIDA subjects. CIMT was generally greater on the left as opposed to the right carotid and was strongly associated with age. When adjustments were made for systolic blood pressure, age, TC:HDL-C it was not possible to demonstrate that this index influenced CIMT in these subjects who were all non-smokers. It is possible that the observation in the MESA study were confounded by smoking, alternatively the number of subjects studied in the MARINA and CRESSIDA studies (311 and 160 respectively) may have been insufficient to detect any effect.

Anderson *et al*. (2009) reported an association between plasma EPA and DHA and subsequent mortality 20 years later. Mozaffarian *et al.* (2013) have recently reported on a prospective cohort in 2692 US adults aged 74 (±5 years) and found a lower risk of death to be associated with high levels of EPA and DHA. The lower risk was largely attributable to few cardiovascular than non-cardiovascular deaths. Individuals in the highest quintile omega-3 PUFA lived on average 2.22 more years after the age of 65 years. These findings, however, do not establish a cause effect relationship because they are subject to residual confounding of other life-style factors.

6.2 Genetic influences on fatty acid biomarkers

A novel finding was the significant influence of additive genetics on the proportion of AA in adipose tissue. Further investigations revealed that variation in *FADS1* SNP, rs174537, explained some but not all of this variation. The association was replicated in plasma lipids from the twins and the similar findings in the MARINA study. An elevated proportion of AA in adipose tissue was noted in one study to be associated with an increased risk of CVD in the review by Harris *et al*. (2007). However, further studies are required to see if this finding with regard to the proportion of AA in adipose tissue is associated with increased risk of CVD can be confirmed. AA besides being a substrate for prostanoid synthesis is also converted into ligands for the endocannabinoid pathway. Anandamide is an amide of AA and arachidonyl glycerol is a powerful agonist of cannabinoid receptors, which are expressed mainly in the brain but also in other tissues including adipose tissue (Pertwee *et al*. 2010). Whether the differences in AA

levels in adipose tissue is of physiological importance is uncertain but it could be an important reservoir of AA. Similarly, the small amounts of DHA in adipose tissue in the context of total body stores. It could be an important reservoir as typically 20-30% of body weight is adipose tissue. DHA is believed to play an important role in the brain and in the retina and there is some uncertainty about the extent to which it synthesised from ALA. The critical period of human development in relation to requirements for DHA is in the last trimester of pregnancy and in early lactation. Human milk contain about 0.2-0.3% of its fatty acids as DHA. Interestingly, this is similar to the level found in adipose tissue. It is possible that variations in *FADS1* and *FADS2* genes may affect levels of LC-PUFA in breast milk and this is an area that could be explored in future studies.

The genetic effect on the delta-5 desaturase metabolites demonstrated by polymorphism rs174537 upstream of the *FADS1* gene appears consistent with some other recent reports (Voruganti *et al*. 2012). Haplotypes carrying three SNP minor alleles were associated with lower D5D and D6D activity, suggesting that any could be in linkage disequilibrium with a functional SNP. However, this study has raised the possibility that another variant on the same haplotype might have more influence on D6D activity than the studied SNPs. In this relatively small sample, a significant interaction between dietary *n*-3 LC-PUFA intake and *FADS1* rs174537 genotype, as a determinant of D5D activity, was demonstrated with potential effects on the composition of LC-PUFA depots and implications for health. Gillingham *et al*. (2013) measured the desaturation

of ALA to LC *n-3* PUFA using stable isotope technique in 26 subjects and related the isotope enrichment rates to variations in rs174545, rs174583, rs174561, and rs174537. They found that carriers of the minor alleles had lower levels of AA and EPA and lower levels of enrichment with isotope following administration of ALA. However, they were unable to demonstrate any differences in enrichment with isotope of DHA, which was very low. A major limitation of this study is very small sample size.

6.3 Conclusion and implications for further research

An important finding from this thesis is that the proportion of SFA in commonly used biomarkers of fatty acid intakes (erythrocyte, plasma total lipids, plasma phospholipids and adipose tissue) are unreliable for assessing relationship between SFA intake and chronic diseases. The use of these biomarkers might be misinterpreted to imply a cause-effect relationship. The Norfolk EPIC cohort is a good example to illustrate this point. This study found branched SFA to be associated with a lower risk of CHD but a low P/S ratio in the same subjects was associated with a higher risk. As branched chain SFA are mainly derived from ruminants, which are rich in SFA, this would suggest that the reason for the relation between a low P/S ratio is a low intake of LA. The author concluded that even chain SFA in plasma phospholipids were positively associated with risk. This information could be claimed to show that SFA increase risk of CHD. However, the authors correctly interpreted their finding as being "consistent with accumulating evidence suggesting a protective role of omega-6 fats substituting for saturated fats for CHD prevention" (Khaw *et al*. 2012).

Biomarkers are probably more reliable for assessing the intake of PUFA than measurement of dietary intake. They appear very sensitive to small changes in the intake of LC *n*-3 PUFA. The measurement in plasma total lipids seems just as reliable as their measurement in phospholipids or erythrocyte lipids in terms of classifying individuals with high, medium or low intakes. A limitation of the genetic studies reported in this thesis is the relative small number of subjects studied. However, there are some very large cohort studies (over 0.5 million participants such as the Bio-bank project and similar projects supported by the Wellcome Trust and the Medical Council) that could be used to assess fatty acid intakes using plasma as a biomarker and then relate to genotype. One lesson learnt from the TwinsUK study is that the volume of blood sample available for analysis is very limited and so it is important to only need very small amounts of plasma or serum for analysis. In this thesis, 0.1 ml plasma was used, but even smaller samples (certainly 50 µL, but possibly as little as 5µL) could be used and robotic techniques (Masood & Salem, 2008) which could speed-up sample handling. Run-time can be shortened using fast chromatography, which employs shorter (12 m), narrow bore columns (0.1 mm) and higher column pressures. However, the run-time reported by Masood & Salem (2008) was 15 min, which is similar to a normal capillary column analysis as used in this thesis using a 0.2 mm x 25 m capillary column. However, the analysis reported in this thesis allowed a longer gap between samples because of the late elution of cholesterol methyl ester, which could contaminate the next run if it was not "burnt off" from the column. The use of split-less inject would increase sensitivity twenty-fold as opposed split injection (used in this thesis) and this approach

with optimising the use of small bore columns and faster responding flame ionisation detectors could possible reduce run-times to below 10 min or possibly 5 min, especially if the late-eluting sterol contaminants could be removed. As an analytical technique, plasma fatty acid composition is relatively inexpensive as the reagents are cheap and the analytical equipment is robust and inexpensive to operate. However, it would be important to have an established quality control procedure to ensure the validity of the assays between different laboratories with test plasma that represent high, medium and low intakes of PUFA or TFA with acceptable performance criteria and appropriate documentation to enable audit.

There would be great merit in applying plasma fatty acid analysis to estimate intakes of fatty acids in populations where food-recording skills are low. It could be applied in order to monitor changes in fatty acid intakes in the diet over time, for example in national dietary surveys such as NDNS. There is also potential for elucidating relationships between PUFA intakes and non-communicable diseases such as cardiovascular disease, type 2 diabetes and cancer which is of great public health importance.

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References

Abbey, M & Nestel, P J (1994). Plasma cholesteryl ester transfer protein activity is increased when trans-elaidic acid is substituted for cis-oleic acid in the diet. *Atherosclerosis,* **106,** 99-107.

Ackman RG, Mag TK (1998). Trans fatty acids and the potential of less in technical products. In: Trans Fatty Acids in Human Nutrition. Editors JL Sebedio and WW Christie. The Oily Press: Dundee.

Andersen, LF, Solvoll, K and Drevon, CA (1996). Very-long-chain n-3 fatty acids as biomarkers for intake of fish and n-3 fatty acid concentrates. *Am J Clin Nutr* **64,** 305-11.

Anderson, SG, Sanders, TA and Cruickshank, JK (2009). Plasma fatty acid composition as a predictor of arterial stiffness and mortality. *Hypertension* **53,** 839-45.

Appel LJ, Brands MW, Daniels SR, Karanja N, Elmer PJ, Sacks FM (2006). Dietary approaches to prevent and treat hypertension: a scientific statement from the American Heart Association, *Hypertension* 47, 296-308.

Arab, L (2003). Biomarkers of fat and fatty acid intake. *J Nutr* **133 Suppl 3,** 925S-932S.

Astorg, P, Bertrais, S, Laporte, F, Arnault, N, Estaquio, C, Galan, P, Favier, A & Hercberg, S (2008). Plasma n-6 and n-3 polyunsaturated fatty acids as biomarkers of their dietary intakes: A cross-sectional study within a cohort of middle-aged french men and women. *Eur J Clin Nutr,* **62,** 1155-61.

Astrup, A, Dyerberg, J, Elwood, P, Hermansen, K, Hu, F B, Jakobsen, M U, Kok, F J, Krauss, R M, Lecerf, J M, Legrand, P, *et al.* (2011). The role of reducing intakes of saturated fat in the prevention of cardiovascular disease: Where does the evidence stand in 2010? *Am J Clin Nutr* **93,** 684-8.

Aulchenko, Y S, Ripatti, S, Lindqvist, I, Boomsma, D, Heid, I M, Pramstaller, P P, Penninx, B W, Janssens, A C, Wilson, J F, Spector, T, *et al.* (2009). Loci influencing lipid levels and coronary heart disease risk in 16 european population cohorts. *Nat Genet* **41,** 47-55.

Baigent, C, Blackwell, L, Emberson, J, Holland, L E, Reith, C, Bhala, N, Peto, R, Barnes, E H, Keech, A, Simes, J, *et al.* (2010). Efficacy and safety of more intensive lowering of LDL cholesterol: A meta-analysis of data from 170,000 participants in 26 randomised trials. *Lancet* **376,** 1670-81.

Bajekal, M, Scholes, S, Love, H, Hawkins, N, O'flaherty, M, Raine, R & Capewell, S (2012). Analysing recent socioeconomic trends in coronary heart disease mortality in england, 2000-2007: A population modelling study. *PLoS Med,* **9** e1001237.

Balk, E M, Lichtenstein, A H, Chung, M, Kupelnick, B, Chew, P & Lau, J (2006). Effects of omega-3 fatty acids on serum markers of cardiovascular disease risk: A systematic review. *Atherosclerosis* **189,** 19-30.

Bang, H O, Dyerberg, J & Sinclair, H M (1980). The composition of the eskimo food in north western greenland. *Am J Clin Nutr,* **33,** 2657-61.

Bartels, M, Cacioppo, J T, Van Beijsterveldt, T C & Boomsma, D I (2013). Exploring the association between well-being and psychopathology in adolescents. *Behav Genet* **43,** 177-90.

Basso, F, Freeman, L, Knapper, C L, Remaley, A, Stonik, J, Neufeld, E B, Tansey, T, Amar, M J, Fruchart-Najib, J, Duverger, N, *et al.* (2003). Role of the hepatic abca1 transporter in modulating intrahepatic cholesterol and plasma hdl cholesterol concentrations. *J Lipid Res,* **44,** 296-302.

Bauer,E, Jakob,s et al. (2005). Principles of physiology of lipid digestion. *Asian-Australasian Journal of Animal Sciences* **18**, 282-295.

Baylin, A, Kabagambe, E K, Ascherio, A, Spiegelman, D & Campos, H (2003). Adipose tissue alpha-linolenic acid and nonfatal acute myocardial infarction in costa rica. *Circulation* **107,** 1586-91.

Baylin, A, Kabagambe, E K, Siles, X & Campos, H (2002). Adipose tissue biomarkers of fatty acid intake. *Am J Clin Nutr* **76,** 750-7.

Baylin, A, Kim, M K, Donovan-Palmer, A, Siles, X, Dougherty, L, Tocco, P & Campos, H (2005). Fasting whole blood as a biomarker of essential fatty acid intake in epidemiologic studies: Comparison with adipose tissue and plasma. *Am J Epidemiol* **162,** 373-81.

Baylin, A, Ruiz-Narvaez, E, Kraft, P & Campos, H (2007). Alpha-Linolenic acid, Delta6-desaturase gene polymorphism, and the risk of nonfatal myocardial infarction. *Am J Clin Nutr* **85,** 554-60.

Bergman, E N, Havel, R J, Wolfe, B M & Bohmer, T (1971). Quantitative studies of the metabolism of chylomicron triglycerides and cholesterol by liver and extrahepatic tissues of sheep and dogs. *J Clin Invest* **50,** 1831-9.

Bernstein, A M, Ding, E L, Willett, W C & Rimm, E B (2012). A meta-analysis shows that docosahexaenoic acid from algal oil reduces serum triglycerides and increases hdl-cholesterol and ldl-cholesterol in persons without coronary heart disease. *J Nutr* **142,** 99-104.

Beynen, A C, Hermus, R J & Hautvast, J G (1980). A mathematical relationship between the fatty acid composition of the diet and that of the adipose tissue in man. *Am J Clin Nutr* **33,** 81-5.

Bingham, S A, Gill, C, Welch, A, Day, K, Cassidy, A, Khaw, K T, Sneyd, M J, Key, T J, Roe, L & Day, N E (1994). Comparison of dietary assessment methods in nutritional epidemiology: Weighed records v. 24 h recalls, food-frequency questionnaires and estimated-diet records. *Br J Nutr* **72,** 619-43.

Bingham, S A, Luben, R, Welch, A, Wareham, N, Khaw, K T & Day, N (2003). Are imprecise methods obscuring a relation between fat and breast cancer? *Lancet,* **362,** 212-4.

Bingham, S A, Welch, A A, Mctaggart, A, Mulligan, A A, Runswick, S A, Luben, R, Oakes, S, Khaw, K T, Wareham, N & Day, N E (2001). Nutritional methods in the european prospective investigation of cancer in norfolk. *Public Health Nutr* **4,** 847-58.

Bokor, S, Dumont, J, Spinneker, A, Gonzalez-Gross, M, Nova, E, Widhalm, K, Moschonis, G, Stehle, P, Amouyel, P, De Henauw, S, *et al.* (2010). Single nucleotide polymorphisms in the fads gene cluster are associated with delta-5 and delta-6 desaturase activities estimated by serum fatty acid ratios. *J Lipid Res,* **51** 2325-33.

Bolton-Smith, C, Woodward, M & Tavendale, R (1997). Evidence for age-related differences in the fatty acid composition of human adipose tissue, independent of diet. *Eur J Clin Nutr* **51,** 619-24.

Boomsma, D, Busjahn, A *et al*. (2002). Classical twin studies and beyond. *Nat Rev Genet* 3 (11), 872-882.

British Nutrition Foundation (1992). Unsaturated fatty acids : nutritional and physiological significance : the report of the British Nutrition Foundation's Task Force, Chapman & Hall.

Brunham, L. R, J. K. Kruit, et al. (2006). Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J Clin Invest* **116,** 1052-1062.

Brunner, E, D. Stallone, et al. (2001). Dietary assessment in Whitehall II: comparison of 7 d diet diary and food-frequency questionnaire and validity against biomarkers. *Br J Nutr* **86**(3), 405-414.

Burdge, G (2004). Alpha-linolenic acid metabolism in men and women: nutritional and biological implications. *Curr Opin Clin Nutr Metab Car*e **7**, 137-144.

Burdge, GC and Calder PC (2005). Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev* **45**, 581-597.

Burr, GO and Burr MM (1929). A new deficiency disease produced by the rigid exclusion of fat from the diet. *J Biol Chem* **82,** 345-367.

Calder, PC (2006). Polyunsaturated fatty acids and inflammation. *Prostaglandins Leukot Essent Fatty Acids* **75**, 197-202.

Campos, H, Baylin, A & Willett, W C (2008). Alpha-linolenic acid and risk of nonfatal acute myocardial infarction. *Circulation* 118, 339-45.

Cantwell, MM, Gibney,MJ *et al*. (2005). Development and validation of a food-frequency questionnaire for the determination of detailed fatty acid intakes. *Public Health Nutr* **8**(1), 97-107.

Chajes, V, Thiebaut, A C, Rotival, M, Gauthier, E, Maillard, V, Boutron-Ruault, M C, Joulin, V, Lenoir, G M & Clavel-Chapelon, F (2008). Association between serum trans-monounsaturated fatty acids and breast cancer risk in the e3n-epic study. *Am J Epidemiol* 167, 1312-20.

Chasman, D I, Pare, G, Mora, S, Hopewell, J C, Peloso, G, Clarke, R, Cupples, L A, Hamsten, A, Kathiresan, S, Malarstig, A, et al. (2009). Forty-three loci associated with plasma lipoprotein size, concentration, and cholesterol content in genome-wide analysis. *PLoS Genet*, 5, e1000730.

Cho, H P, Nakamura, M & Clarke, S D (1999a). Cloning, expression, and fatty acid regulation of the human delta-5 desaturase. *J Biol Chem* 274, 37335-9.

Cho, H P, Nakamura, M T & Clarke, S D (1999b). Cloning, expression, and nutritional regulation of the mammalian delta-6 desaturase. *J Biol Chem* 274, 471-7.

Christie WW. (1990). Gas chromatography and lipids. Bridgewater, Oily Press. Available at: www.lipid.co.uk

Christie, W. W. and X. Han (2010). Lipid analysis : isolation, separation, identification and lipidomic analysis. Bridgwater, Oily Press.

Clarke, R, Shipley, M, Armitage, J, Collins, R & Harris, W (2009). Plasma phospholipid fatty acids and chd in older men: Whitehall study of London civil servants. *Br J Nutr* **102**, 279-84.

Clifton, P M, Keogh, J B & Noakes, M (2004). Trans fatty acids in adipose tissue and the food supply are associated with myocardial infarction. *J Nutr* **134**, 874-9.

Cobley, L. S. and W. M. Steele (1976). An introduction to the botany of tropical crops. London, Longman.

Coleman, M P, Key, T J, Wang, D Y, Hermon, C, Fentiman, I S, Allen, D S, Jarvis, M, Pike, M C & Sanders, T A (1992). A prospective study of obesity, lipids, apolipoproteins and ischaemic heart disease in women. *Atherosclerosis* **92**, 177-85.

Coppack, S W, Evans, R D, Fisher, R M, Frayn, K N, Gibbons, G F, Humphreys, S M, Kirk, M L, Potts, J L & Hockaday, T D (1992). Adipose tissue metabolism in obesity: Lipase action in vivo before and after a mixed meal. *Metabolism* 41, 264-72.

Craig, LC, Thies, F et al (2009). Relative validity of fatty acid intakes from an FFQ compared with subcutaneous adipose tissue fatty acids. *Proc Nutr Soc* **68 (OCE)**, E92.

Crowe, F L, Appleby, P N, Travis, R C & Key, T J (2013). Risk of hospitalization or death from ischemic heart disease among british vegetarians and nonvegetarians: Results from the epic-oxford cohort study. *Am J Clin Nutr* 97, 597-603.

Das, UN. (2008). Essential fatty acids and their metabolites could function as endogenous HMG-CoA reductase and ACE enzyme inhibitors, anti-arrhythmic, anti-hypertensive, anti-atherosclerotic, anti-inflammatory, cytoprotective, and cardioprotective molecules. *Lipids Health Dis* **7**: 37.

Davey, G K, Spencer, E A, Appleby, P N, Allen, N E, Knox, K H & Key, T J (2003). Epic-oxford: Lifestyle characteristics and nutrient intakes in a cohort of 33 883 meat-eaters and 31 546 non meat-eaters in the UK. *Public Health Nutr*, 6, 259-69.

Dayton, S, S. Hashimoto, et al. (1966). Composition of lipids in human serum and adipose tissue during prolonged feeding of a diet high in unsaturated fat. *J Lipid Res* **7**(1), 103-111.

Department of Health (1991). Dietary reference values for food energy and nutrients for the United Kingdom: Report of the Panel on Dietary Reference Values of the Committee on Medical Aspects of Food Policy. London: HMSO.

Department of Health (1994). Nutritional aspects of cardiovascular disease : report of the Cardiovascular Review Group Committee on Medical Aspects of Food Policy. London, HMSO.

Dietary Guidelines for Americans (2010). http://www.health.gov/dietaryguidelines/ [accessed 7/7/2013].

Dixon, A L, Liang, L, Moffatt, M F, Chen, W, Heath, S, Wong, K C, Taylor, J, Burnett, E, Gut, I, Farrall, M, *et al.* (2007). A genome-wide association study of global gene expression. *Nat Genet* 39, 1202-7.

Drazen, JM (2005). COX-2 inhibitors--a lesson in unexpected problems. *N Engl J Med* **352**(11), 1131-1132.

Durrington, P N, Bolton, C H, Hartog, M, Angelinetta, R, Emmett, P & Furniss, S (1977). The effect of a low-cholesterol, high-polyunsaturate diet on serum lipid levels, apolipoprotein b levels and triglyceride fatty acid composition. *Atherosclerosis* 27, 465-75.

Dyerberg, J, Bang, H O, Stoffersen, E, Moncada, S & Vane, J R (1978). Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis? *Lancet* 2, 117-9.

Eastwood, M (2009). 'Evaluation of dietary intake', In Principles Of Human Nutrition. 2nd ed. Wiley, pp.123-131.

EFSA (2010). EFSA Panel on Dietetic Products, Nutrition, and Allergies (NDA). 'Scientific Opinion on Dietary Reference Values for Fats, Including Saturated Fatty Acids, Polyunsaturated Fatty Acids, Monounsaturated Fatty Acids, Trans Fatty Acids, and Cholesterol', *EFSA Journal* **8(3),1461**.

Fahy, E, Subramaniam, S, Brown, H A, Glass, C K, Merrill, A H, Jr, Murphy, R C, Raetz, C R, Russell, D W, Seyama, Y, Shaw, W, et al. (2005). A comprehensive classification system for lipids. *J Lipid Res* 46, 839-61.

Flachs, P, Rossmeisl, M, Bryhn, M & Kopecky, J (2009). Cellular and molecular effects of n-3 polyunsaturated fatty acids on adipose tissue biology and metabolism. *Clin Sci (Lond)* 116, 1-16.

Food and Agriculture Organization of the United Nations. (2010). Fats and fatty acids in human nutrition : report of an expert consultation : 10-14 November 2008, Geneva. Rome, Food and Agriculture Organization of the United Nations.

Frayn K and Stanner S (2005) 'The aetology and epidemiology of cardiovascular disease', in Stanner S. (ed.) Report of the British Nutrition Foundation Task Force on Cardiovascular disease: diet, nutrition and emerging risk factors. pp. 1-21 Oxford: Blackwell.

Fuhrman, B J, Barba, M, Krogh, V, Micheli, A, Pala, V, Lauria, R, Chajes, V, Riboli, E, Sieri, S, Berrino, F, et al. (2006). Erythrocyte membrane phospholipid composition as a biomarker of dietary fat. *Ann Nutr Metab*, 50, 95-102.

Garcia-Fernandez, M, Gutierrez-Gil, B, Garcia-Gamez, E & Arranz, J J (2009). Genetic variability of the stearoyl-coa desaturase gene in sheep. *Mol Cell Probes* 23, 107-11.

Garland, M, Sacks, FM, Colditz, GA, Rimm, E B, Sampson, L A, Willett, W C & Hunter, D J (1998). The relation between dietary intake and adipose tissue composition of selected fatty acids in us women. *Am J Clin Nutr* 67, 25-30.

Gaunt, TR, Rodriguez, S & Day, IN (2007). Cubic exact solutions for the estimation of pairwise haplotype frequencies: Implications for linkage disequilibrium analyses and a web tool 'cubex'. *BMC Bioinformatics* 8, 428.

Gillingham, L G, Harding, S V, Rideout, T C, Yurkova, N, Cunnane, S C, Eck, P K & Jones, P J (2013). Dietary oils and fads1-fads2 genetic variants modulate [13c]alpha-linolenic acid metabolism and plasma fatty acid composition. *Am J Clin Nutr* **97**, 195-207.

Glaser, C, Lattke, E, Rzehak, P, Steer, C & Koletzko, B (2011). Genetic variation in polyunsaturated fatty acid metabolism and its potential relevance for human development and health. *Matern Child Nutr* **7 Supp 2**: 27-40.

Godley, P A, Campbell, M K, Miller, C, Gallagher, P, Martinson, F E, Mohler, J L & Sandler, R S (1996). Correlation between biomarkers of omega-3 fatty acid consumption and questionnaire data in african american and caucasian united states males with and without prostatic carcinoma. *Cancer Epidemiol Biomarkers Prev* 5, 115-9.

Goldstein, J L & Brown, M S (2009). The LDL receptor. *Arterioscler Thromb Vasc Biol* 29, 431-8.

Goodnight, S H, Jr., Harris, W S, Connor, W E & Illingworth, D R (1982). Polyunsaturated fatty acids, hyperlipidemia, and thrombosis. *Arteriosclerosis* **2**, 87-113.

Goyens, P L, Spilker, M E, Zock, P L, Katan, M B & Mensink, R P (2006). Conversion of alpha-linolenic acid in humans is influenced by the absolute amounts of alpha-linolenic acid and linoleic acid in the diet and not by their ratio. *Am J Clin Nutr* **84**, 44-53.

Guillou, H, Zadravec, D, Martin, P G & Jacobsson, A (2010). The key roles of elongases and desaturases in mammalian fatty acid metabolism: Insights from transgenic mice. *Prog Lipid Res* **49**, 186-99.

Gurr, MI, Harwood, JL, and Frayne KN (2002). Lipid biochemistry. Malden, Mass, Blackwell Science.

Hammond, CJ, Snieder, H, Gilbert, CE & Spector, TD (2001). Genes and environment in refractive error: the twin eye study. *Invest Ophthalmol Vis Sci* **42,** 1232-6.

Harris, W S, Poston, W C & Haddock, C K (2007). Tissue n-3 and n-6 fatty acids and risk for coronary heart disease events. *Atherosclerosis* 193, 1-10.

Hayes, K C & Khosla, P (1992). Dietary fatty acid thresholds and cholesterolemia. *FASEB* **J 6**, 2600-7.

He, K, Liu, K, Daviglus, M L, Jenny, N S, Mayer-Davis, E, Jiang, R, Steffen, L, Siscovick, D, Tsai, M & Herrington, D (2009). Associations of dietary long-chain n-3 polyunsaturated fatty acids and fish with biomarkers of inflammation and endothelial activation (from the multi-ethnic study of atherosclerosis [mesa]). *Am J Cardiol* **103**, 1238-43.

Hegsted, D M, Mcgandy, R B, Myers, M L & Stare, F J (1965). Quantitative effects of dietary fat on serum cholesterol in man. *Am J Clin Nutr* **17**, 281-95.

Hellerstein, MK (1999). De novo lipogenesis in humans: metabolic and regulatory aspects. *Eur J Clin Nutr* **53 Suppl 1**: S53-65.

Hellstrand, S, Sonestedt, E, Ericson, U, Gullberg, B, Wirfalt, E, Hedblad, B & Orho-Melander, M (2012). Intake levels of dietary long-chain PUFAs modify the association between genetic variation in FADS and LDL-C. *J Lipid Res* **53**, 1183-9.

Henderson L, Gregory J, Irving K et al. (2003) The National Diet & Nutrition Survey: adults aged 19 to 64 years. Vol. 2: Energy, protein, carbohydrate, fat and alcohol intake. London: HMSO. http://www.food.gov.uk/multimedia/pdfs/ndnsv2.pdf.

Hippisley-Cox J, Coupland C, Vinogradova Y, Robson J, May M, Brindle P (2007). Derivation and validation of QRISK, a new cardiovascular disease risk score for the United Kingdom: prospective open cohort study. *BMJ* **335**(7611), 136-

Hodge, A M, Simpson, J A, Gibson, R A, Sinclair, A J, Makrides, M, O'dea, K, English, D R & Giles, G G (2007). Plasma phospholipid fatty acid composition as a biomarker of habitual dietary fat intake in an ethnically diverse cohort. *Nutr Metab Cardiovasc Dis* 17, 415-26.

Hodson, L, Skeaff, CM, Fielding, BA (2008). Fatty acid composition of adipose tissue and blood in humans and its use as a biomarker of dietary intake. *Prog Lipid Res* **47**(5), 348-380.

Holland, B., Welch, A.A., Unwin, I.D., Buss, D.H., Paul, A.A. & Southgate, D.A.T. (1991) McCance and Widdowson's The Composition of Foods. Fifth Revised and Extended Edition. Cambridge and London: The Royal Society of Chemistry and Ministry of Agriculture, Fisheries and Food.

Homeostasis Model Assessment (HOMA), a software implementation of the HOMA2 model developed by Diabetes Trials Unit, University of Oxford http://www.dtu.ox.ac.uk/homacalculator/ [access 7/7/2013]

Hooper, L, Abdelhamid, A, Moore, H J, Douthwaite, W, Skeaff, C M & Summerbell, C D (2012). Effect of reducing total fat intake on body weight: Systematic review and meta-analysis of randomised controlled trials and cohort studies. *BMJ*, 345, e7666.

Hooper, L, Summerbell, C D, Thompson, R, Sills, D, Roberts, F G, Moore, H J & Davey Smith, G (2012). Reduced or modified dietary fat for preventing cardiovascular disease. *Cochrane Database Syst Rev*, **5,** CD002137.

Illig, T, Gieger, C, Zhai, G, Romisch-Margl, W, Wang-Sattler, R, Prehn, C, Altmaier, E, Kastenmuller, G, Kato, B S, Mewes, H W, et al. (2010). A genome-wide perspective of genetic variation in human metabolism. *Nat Genet* **42**, 137-41.

International Diabetes Federation (2006) The IDF consensus worldwide definition of the metabolic syndrome [article online]. Available at: http://www.idf.org/ [accessed 7/7/2013].

Jakobsen, M U, O'reilly, E J, Heitmann, B L, Pereira, M A, Balter, K, Fraser, G E, Goldbourt, U, Hallmans, G, Knekt, P, Liu, S, *et al.* (2009). Major types of dietary fat and risk of coronary heart disease: A pooled analysis of 11 cohort studies. *Am J Clin Nutr*, **89,** 1425-32.

Jebb, S A, Lovegrove, J A, Griffin, B A, Frost, G S, Moore, C S, Chatfield, M D, Bluck, L J, Williams, C M, Sanders, T A & Risck Study Group (2010). Effect of changing the amount and type of fat and carbohydrate on insulin sensitivity and cardiovascular risk: The RISCK (Reading, Imperial, Surrey, Cambridge, and Kings) trial. *Am J Clin Nutr*, **92,** 748-758.

Jensen, C L, Prager, T C, Fraley, J K, Chen, H, Anderson, R E & Heird, W C (1997). Effect of dietary linoleic/alpha-linolenic acid ratio on growth and visual function of term infants. *J Pediatr* 131, 200-9.

Jeppesen, C, Jorgensen, M E & Bjerregaard, P (2012). Assessment of consumption of marine food in greenland by a food frequency questionnaire and biomarkers. *Int J Circumpolar Health*, 71 18361.

Jump, D B (2011). Fatty acid regulation of hepatic lipid metabolism. *Curr Opin Clin Nutr Metab Care* **14,** 115-20.

Katan, M B, Deslypere, J P, Van Birgelen, A P, Penders, M & Zegwaard, M (1997). Kinetics of the incorporation of dietary fatty acids into serum cholesteryl esters, erythrocyte membranes, and adipose tissue: An 18-month controlled study. *J Lipid Res* **38**, 2012-22.

Kathiresan, S, Willer, C J, Peloso, G M, Demissie, S, Musunuru, K, Schadt, E E, Kaplan, L, Bennett, D, Li, Y, Tanaka, T, et al. (2009). Common variants at 30 loci contribute to polygenic dyslipidemia. *Nat Genet* 41, 56-65.

Key, TJ, Appleby, PN et al. (2009). Mortality in British vegetarians: results from the European Prospective Investigation into Cancer and Nutrition (EPIC-Oxford). *Am J Clin Nutr* **89**(5), 1613S-1619S.

Keys, A, Anderson, J T & Grande, F (1957). Prediction of serum-cholesterol responses of man to changes in fats in the diet. *Lancet* **273**, 959-66.

Keys, A, Menotti, A, Karvonen, M J, Aravanis, C, Blackburn, H, Buzina, R, Djordjevic, B S, Dontas, A S, Fidanza, F, Keys, M H, et al. (1986). The diet and 15-year death rate in the seven countries study. *Am J Epidemiol* **124**, 903-15.

Keys, ANCE and Parlin RW (1966). Serum Cholesterol Response to Changes in Dietary Lipids. *Am J Clin Nutr* **19**, 175-181.

Kgwatalala, P M, Ibeagha-Awemu, E M, Hayes, J F & Zhao, X (2007). Single nucleotide polymorphisms in the open reading frame of the stearoyl-coa desaturase gene and resulting genetic variants in Canadian Holstein and Jersey cows. *DNA Seq* 18, 357-62.

Khaw, K T, Friesen, M D, Riboli, E, Luben, R & Wareham, N (2012). Plasma phospholipid fatty acid concentration and incident coronary heart disease in men and women: The EPIC-Norfolk prospective study. *PLoS Med,* **9,** e1001255.

Khoury, MJ, Beaty, TH et al. (1993). Fundamentals of genetic epidemiology. New York ; Oxford, Oxford University Press.

Koletzko, B, Knoppke, B, Von Schenck, U, Demmelmair, H & Damli, A (1999). Noninvasive assessment of essential fatty acid status in preterm infants by buccal mucosal cell phospholipid analysis. *J Pediatr Gastroenterol Nutr* 29, 467-74.

Kopecky, J, Rossmeisl, M, Flachs, P, Kuda, O, Brauner, P, Jilkova, Z, Stankova, B, Tvrzicka, E & Bryhn, M (2009). N-3 pufa: Bioavailability and modulation of adipose tissue function. *Proc Nutr Soc* 68, 361-9.

Kuratko, CN and Salem, NJr. (2009). Biomarkers of DHA status. *Prostaglandins Leukot Essent Fatty Acids* **81**(2-3), 111-118.

Laitinen, K, Sallinen, J, Linderborg, K & Isolauri, E (2006). Serum, cheek cell and breast milk fatty acid compositions in infants with atopic and non-atopic eczema. *Clin Exp Allergy* **36,** 166-73.

Lattka, E, Eggers, S, Moeller, G, Heim, K, Weber, M, Mehta, D, Prokisch, H, Illig, T & Adamski, J (2010). A common FADS2 promoter polymorphism increases promoter activity and facilitates binding of transcription factor elk1. *J Lipid Res* **51**, 182-91.

Lattka, E, Illig, T, Heinrich, J & Koletzko, B (2009). FADS gene cluster polymorphisms: Important modulators of fatty acid levels and their impact on atopic diseases. *J Nutrigenet Nutrigenomics* **2**, 119-28.

Lattka, E, Rzehak, P, Szabo, E, Jakobik, V, Weck, M, Weyermann, M, Grallert, H, Rothenbacher, D, Heinrich, J, Brenner, H, et al. (2011). Genetic variants in the FADS gene cluster are associated with arachidonic acid concentrations of human breast milk at 1.5 and 6 mo postpartum and influence the course of milk dodecanoic, tetracosenoic, and trans-9-octadecenoic acid concentrations over the duration of lactation. *Am J Clin Nutr* 93, 382-91.

Lattka, E, T. Illig, et al. (2010). Genetic variants of the FADS1 FADS2 gene cluster as related to essential fatty acid metabolism. *Curr Opin Lipidol* **21**(1), 64-69.

Leat, W M, Northrop, C A, Harrison, F A & Cox, R W (1983). Effect of dietary linoleic and linolenic acids on testicular development in the rat. *Q J Exp Physiol* **68**, 221-31.

Lee, J, Chen, L, Snieder, H, Chen Da, F, Lee, L M, Liu, G F, Wu, T, Tang, X, Zhan, S Y, Cao, W H, et al. (2010). Heritability of obesity-related phenotypes and association with adiponectin gene polymorphisms in the chinese national twin registry. *Ann Hum Genet* 74, 146-54.

Lemaitre, R N, King, I B, Mozaffarian, D, Sotoodehnia, N, Rea, T D, Kuller, L H, Tracy, R P & Siscovick, D S (2006). Plasma phospholipid trans fatty acids, fatal ischemic heart disease, and sudden cardiac death in older adults: The Cardiovascular Health Study. *Circulation* 114, 209-15.

Lemaitre, R N, Tanaka, T, Tang, W, Manichaikul, A, Foy, M, Kabagambe, E K, Nettleton, J A, King, I B, Weng, L C, Bhattacharya, S, et al. (2011). Genetic loci associated with plasma phospholipid n-3 fatty acids: A meta-analysis of genome-wide association studies from the CHARGE consortium. *PLoS Genet* 7, e1002193.

Lepage, G & Roy, C C (1986). Direct transesterification of all classes of lipids in a one-step reaction. *J Lipid Res* 27, 114-20.

Lewington, S *et al*. (2007). Blood cholesterol and vascular mortality by age, sex, and blood pressure: A meta-analysis of individual data from 61 prospective studies with 55 000 vascular deaths. *Lancet*, **370,** 1829-1839.

Leyton, J, Drury, P J & Crawford, M A (1987). Differential oxidation of saturated and unsaturated fatty acids in vivo in the rat. *Br J Nutr* 57 383-93.

Lloyd-Wright, Z, T. J. A. Key, et al. (2003). Plasma conjugated linoleic acid concentrations in vegan, vegetarian and omnivore men recruited into the EPIC study: 9-cis, 11 trans octadecadienoic acid are markers for the intake of ruminant derived fat. *Proc Nutr Soc* **62**(OCA-B), 42A-42A.

Lloyd-Wright, Z (2005). The nutritional status of vegan men in the United Kingdom with particular reference to vitamin B12. PhD thesis, King's College London.

Lu, Y, Feskens, E J, Dolle, M E, Imholz, S, Verschuren, W M, Muller, M & Boer, J M (2010). Dietary n-3 and n-6 polyunsaturated fatty acid intake interacts with FADS1 genetic variation to affect total and HDL-cholesterol concentrations in the Doetinchem Cohort Study. *Am J Clin Nutr* **92**, 258-65.

Ma, J, Folsom, A R, Shahar, E & Eckfeldt, J H (1995). Plasma fatty acid composition as an indicator of habitual dietary fat intake in middle-aged adults. The Atherosclerosis Risk In Communities (ARIC) study investigators. *Am J Clin Nutr* **62**, 564-71.

Macgregor, A J, Snieder, H, Schork, N J & Spector, T D (2000). Twins. Novel uses to study complex traits and genetic diseases. *Trends Genet* 16, 131-4.

MAFF (1991). Household food consumption and expenditure 1990 with a study of trends over the period 1940-1990. Annual Report of the Nutrional Food Survey Committee. London, HMSO.

MAFF Ministry of Agriculture Fisheries and Food. (1998) Fatty acids. Seventh supplement to the Fifth Edition of McCance and Widdowson's The Composition of Foods. Cambridge and London: The Royal Society of Chemistry and Ministry of Agriculture, Fisheries and Food.

Malerba, G, Schaeffer, L, Xumerle, L, Klopp, N, Trabetti, E, Biscuola, M, Cavallari, U, Galavotti, R, Martinelli, N, Guarini, P, et al. (2008). Snps of the fads gene cluster are associated with polyunsaturated fatty acids in a cohort of patients with cardiovascular disease. *Lipids* **43**, 289-99.

Marangoni, A. G. and D. Rousseau (1995). Engineering Triacylglycerols - the Role of Interesterification. *Trends in Food Science & Technology* **6**(10), 329-335.

Marckmann, P, A. Lassen, et al. (1995). Biomarkers of habitual fish intake in adipose tissue. *Am J Clin Nutr* **62**(5), 956-959.

Martinelli, N, Girelli, D, Malerba, G, Guarini, P, Illig, T, Trabetti, E, Sandri, M, Friso, S, Pizzolo, F, Schaeffer, L, et al. (2008). FADS genotypes and desaturase activity estimated by the ratio of arachidonic acid to linoleic acid are associated with inflammation and coronary artery disease. *Am J Clin Nutr* **88**, 941-9.

Masood, M. A. and N. Salem, Jr. (2008). High-throughput analysis of plasma fatty acid methyl esters employing robotic transesterification and fast gas chromatography. *Lipids* **43**(2), 171-180.

McCaffery, J M, Snieder, H, Dong, Y & De Geus, E (2007). Genetics in psychosomatic medicine: Research designs and statistical approaches. *Psychosom Med* **69**, 206-16.

McCance, R. A. and E. M. Widdowson (1998). Fatty acids : seventh supplement to the fifth edition of McCance and Widdowson's The composition of foods. Cambridge, Royal Society of Chemistry.

McCrorie TA, Keaveney EM, Wallace JM, Binns N, Livingstone MB. (2011). Human health effects of conjugated linoleic acid from milk and supplements. *Nutr Res Rev* **24**(2),206-27.

Mcdevitt, R M, Bott, S J, Harding, M, Coward, W A, Bluck, L J & Prentice, A M (2001). De novo lipogenesis during controlled overfeeding with sucrose or glucose in lean and obese women. *Am J Clin Nutr* **74**, 737-46.

Mcmurchie, E J, Margetts, B M, Beilin, L J, Croft, K D, Vandongen, R & Armstrong, B K (1984). Dietary-induced changes in the fatty acid composition of human cheek cell phospholipids: Correlation with changes in the dietary polyunsaturated/saturated fat ratio. *Am J Clin Nutr* **39**, 975-80.

Mensink, R P, Zock, P L, Kester, A D & Katan, M B (2003). Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: A meta-analysis of 60 controlled trials. *Am J Clin Nutr,* **77,** 1146-1155.

Merlin (Multiple Engine for Rapid Likelihood Inference) software (www.genomeutwin.org/member/cores.stat/linkage/merlin.html [accessed 4/7/2013].

Metz, J G, Roessler, P, Facciotti, D, Levering, C, Dittrich, F, Lassner, M, Valentine, R, Lardizabal, K, Domergue, F, Yamada, A, et al. (2001). Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**, 290-3.

Milanesi, E, Nicoloso, L & Crepaldi, P (2008). Stearoyl CoA desaturase (SCD) gene polymorphisms in Italian cattle breeds. *J Anim Breed Genet* **125**, 63-7.

Mohammed, I, Cherkas, LF, Riley, SA, Spector, TD & Trudgill, NJ (2005). Genetic influences in irritable bowel syndrome: a twin study. *Am J Gastroenterol* **100,** 1340-4.

Molto-Puigmarti, C, Plat, J, Mensink, R P, Muller, A, Jansen, E, Zeegers, M P & Thijs, C (2010). FADS1 FADS2 gene variants modify the association between fish intake and the docosahexaenoic acid proportions in human milk. *Am J Clin Nutr* **91**, 1368-76.

Moore, R A, Oppert, S, Eaton, P & Mann, J I (1977). Triglyceride fatty acids confirm a change in dietary fat. *Clin Endocrinol (Oxf)* 7, 143-9.

Mozaffarian, D & Clarke, R (2009). Quantitative effects on cardiovascular risk factors and coronary heart disease risk of replacing partially hydrogenated vegetable oils with other fats and oils. *Eur J Clin Nutr* **63 Suppl 2,** S22-33.

Mozaffarian, D, Ascherio, A, Hu, F B, Stampfer, M J, Willett, W C, Siscovick, D S & Rimm, E B (2005). Interplay between different polyunsaturated fatty acids and risk of coronary heart disease in men. *Circulation* **111**, 157-64.

Mozaffarian, D, Lemaitre, R N, King, I B, Song, X, Huang, H, Sacks, F M, Rimm, E B, Wang, M & Siscovick, D S (2013). Plasma phospholipid long-chain omega-3 fatty acids and total and cause-specific mortality in older adults: A cohort study. *Ann Intern Med* **158**, 515-25.

Nakamura, MT and Nara, TY (2004). Structure, function, and dietary regulation of delta6, delta5, and delta9 desaturases. *Annu Rev Nutr* **24**, 345-376.

Nakayama, K, T. Bayasgalan, et al. (2010). A single nucleotide polymorphism in the FADS1/FADS2 gene is associated with plasma lipid profiles in two genetically similar Asian ethnic groups with distinctive differences in lifestyle. *Hum Genet* **127**(6), 685-690.

NCEP-3 (2001). Executive Summary of the Third Report of the National Cholesterol Education Program. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). JAMA **285**: 2486–2497.

Neale, M. C, Boker, S. M, Xie, G, & Maes, H H (2002). *Mx: Statistical Modeling* (6th ed). Richmond,VA: Medical College of Virginia, Department of Psychiatry.

Nelson M and Bingham S. (1997) 'Assessment of food consumption and nutrient intake', in Margetts B and Nelson M. (eds.) Design concept in nutritional epidemiology. 2nd ed. Oxford: Oxford University Press, pp. 123-169

Paton, CM and Ntambi, JM (2009). Biochemical and physiological function of stearoyl-CoA desaturase. *Am J Physiol Endocrinol Metab* **297**, E28-37.

Pertwee, RG, Howlett, AC *et al*. (2010). International Union of Basic and Clinical Pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB(1) and CB(2). *Pharmacol Rev* **62**, 588-631.

Rader, DJ and Daugherty, A (2008). Translating molecular discoveries into new therapies for atherosclerosis. *Nature* **451**(7181), 904-913.

Ratnayake, WM and Galli, C (2009). Fat and fatty acid terminology, methods of analysis and fat digestion and metabolism: a background review paper. *Ann Nutr Metab* **55**(1-3), 8-43.

Rosell MS, Lloyd-Wright Z, et al. (2005). Long-chain n-3 polyunsaturated fatty acids in plasma in British meat-eating, vegetarian, and vegan men. *Am J ClinNutr* **82**(2),327-34.

Roshanai, F & Sanders, T A (1985). Influence of different supplements of n-3 polyunsaturated fatty acids on blood and tissue lipids in rats receiving high intakes of linoleic acid. *Ann Nutr Metab,* **29,** 189-96.

Ross, R. (1999). Atherosclerosis-an inflammatory disease. *N Engl J Med* **340**(2), 115-126.

Saadatian-Elahi, M, N. Slimani, et al. (2009). Plasma phospholipid fatty acid profiles and their association with food intakes: results from a cross-sectional study within the European Prospective Investigation into Cancer and Nutrition. *Am J Clin Nutr* **89**(1), 331-346.

Sabatti, C, S. K. Service, et al. (2009). Genome-wide association analysis of metabolic traits in a birth cohort from a founder population. *Nat Genet* **41**(1), 35-46.

Sanders TA, Gleason K, Griffin B, *et al.* (2006). Influence of an algal triacylglycerol containing docosahexaenoic acid (22 : 6n-3) and docosapentaenoic acid (22 : 5n-6) on cardiovascular risk factors in healthy men and women. *Br J Nutr* 95(3),525-31.

Sanders, TA (1988). Essential and trans-Fatty acids in nutrition. *Nutr Res Rev* **1**(1), 57-78.

Sanders, T A (2009a). Fat and fatty acid intake and metabolic effects in the human body. *Ann Nutr Metab* **55,** 162-72.

Sanders, TA, Gleason, K, Griffin, B & Miller, GJ (2006b). Influence of an algal triacylglycerol containing docosahexaenoic acid (22 : 6n-3) and docosapentaenoic acid (22 : 5n-6) on cardiovascular risk factors in healthy men and women. *Br J Nutr,* **95,** 525-31.

Sanders, TA, Lewis, F, Slaughter, S, Griffin, BA, Griffin, M, Davies, I, Millward, DJ, Cooper, JA & Miller, GJ (2006a). Effect of varying the ratio of n-6 to n-3 fatty acids by increasing the dietary intake of alpha-linolenic acid, eicosapentaenoic and docosahexaenoic acid, or both on fibrinogen and clotting factors VII and XII in persons aged 45-70 y: The OPTILIP study. *Am J Clin Nutr,* **84,** 513-22.

Sanders, T and Emery P (2003). Molecular basis of human nutrition. London ; New York, Taylor & Francis.

Sanders, TA (2009b). DHA status of vegetarians. *Prostaglandins Leukot Essent Fatty Acids,* **81,** 137-41.

Sanders, TA and Naismith DJ (1980). The metabolism of alpha-linolenic acid by the foetal rat. *Br J Nutr* **44**(2), 205-208.

Sanders, TA and SE Berry SE (2005). Influence of stearic acid on postprandial lipemia and hemostatic function. *Lipids* **40**(12), 1221-1227.

Sanders, TA, Ellis, F R & Dickerson, JW (1978). Studies of vegans: The fatty acid composition of plasma choline phosphoglycerides, erythrocytes, adipose tissue, and breast milk, and some indicators of susceptibility to ischemic heart disease in vegans and omnivore controls. *Am J Clin Nutr,* **31,** 805-13.

Sanders, TA, Hall, WL, Maniou, Z, Lewis, F, Seed, PT & Chowienczyk, PJ (2011). Effect of low doses of long-chain n-3 pufas on endothelial function and arterial stiffness: A randomized controlled trial. *Am J Clin Nutr* **94,** 973-80.

Sanders, TA, Oakley, FR *et al*. (1997). Influence of n-6 versus n-3 polyunsaturated fatty acids in diets low in saturated fatty acids on plasma lipoproteins and hemostatic factors. *Arterioscler Thromb Vasc Biol* **17**(12), 3449-3460.

Sanders, TA, Oakley, FR, Crook, D, Cooper, JA & Miller, GJ (2003). High intakes of trans monounsaturated fatty acids taken for 2 weeks do not influence procoagulant and fibrinolytic risk markers for chd in young healthy men. *Br J Nutr,* **89,** 767-76.

Sanders, TA, Vickers, et al. (1981). Effect on blood lipids and haemostasis of a supplement of cod-liver oil, rich in eicosapentaenoic and docosahexaenoic acids, in healthy young men. *Clin Sci (Lond)* **61**(3), 317-324.

Sanders, TAB, Mistry, M et al. (1984). The Influence of a Maternal Diet Rich in Linoleic-Acid on Brain and Retinal Docosahexaenoic Acid in the Rat. *Br J Nutr* **51**(1), 57-66.

Sanderson, P, Finnegan, YE et al. (2002). UK Food Standards Agency alpha-linolenic acid workshop report. *Br J Nutr* **88**(5), 573-579.

Sarwar, N, Sandhu,MS et al. (2010). Triglyceride-mediated pathways and coronary disease: collaborative analysis of 101 studies. *Lancet* **375**(9726), 1634-1639.

Schaeffer, L, Gohlke,h et al. (2006). Common genetic variants of the FADS1 FADS2 gene cluster and their reconstructed haplotypes are associated with the fatty acid composition in phospholipids. *Hum Mol Genet* **15**(11), 1745-1756.

Sebedio, JL and Christie,WW(1998). Trans fatty acids in human nutrition. Dundee, Oily Press.

Seppanen-Laakso, T, Laakso, I, Vanhanen, H, Kiviranta, K, Lehtimaki, T & Hiltunen, R (2001). Major human plasma lipid classes determined by quantitative high-performance liquid

chromatography, their variation and associations with phospholipid fatty acids. *J Chromatogr B: Biomed Sci Appl*, 754, 437-45.

Serhan, C. N, K. Gotlinger, et al. (2004). Resolvins, docosatrienes, and neuroprotectins, novel omega-3-derived mediators, and their aspirin-triggered endogenous epimers: an overview of their protective roles in catabasis. *Prostaglandins Other Lipid Mediat* **73(**3-4), 155-172.

Shah SH, Hauser ER, Bain JR *et al*. (2009). High heritability of metabolomic profiles in families burdened with premature cardiovascular disease. *Mol Syst Biol* **5**: 258.

Siguel EN, Chee KM, Gong JX, Schaefer EJ (1987). Criteria for Essential Fatty-Acid Deficiency in Plasma as Assessed by Capillary Column Gas-Liquid-Chromatography. *Clin Chem* **33**(10), 1869-1873.

Sim PY (2011). The effects of Different Types of Dietary Fats in the Liver. PhD Thesis, King's College London.

Simopoulos, AP (2011). Importance of the omega-6/omega-3 balance in health and disease: evolutionary aspects of diet. *World Rev Nutr Diet* **102**: 10-21.

Siri-Tarino, P W, Sun, Q, Hu, F B & Krauss, R M (2010). Meta-analysis of prospective cohort studies evaluating the association of saturated fat with cardiovascular disease. *Am J Clin Nutr*, **91,** 535-46.

Skeaff, CM and Miller J (2009). Dietary fat and coronary heart disease: summary of evidence from prospective cohort and randomised controlled trials. *Ann Nutr Metab* **55**(1-3), 173-201.

Smolina, K, Wright, F L, Rayner, M & Goldacre, M J (2012). Determinants of the decline in mortality from acute myocardial infarction in england between 2002 and 2010: Linked national database study. *BMJ*, **344,** d8059.

Spector, TD and Williams, FM (2006). The UK Adult Twin Registry (TwinsUK). *Twin Res Hum Genet* **9**(6), 899-906.

Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL.. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* **320**(14), 915-924.

Stewart, M. E, M. W. McDonnell, et al. (1986). Possible genetic control of the proportions of branched-chain fatty acids in human sebaceous wax esters. *J Invest Dermatol* 86(6), 706-708.

Strawford, A, F. Antelo, et al. (2004). Adipose tissue triglyceride turnover, de novo lipogenesis, and cell proliferation in humans measured with (H2O)-H-2. *Am J Physiol Endocrinol Metabol* **286**(4), E577-E588.

Sullivan BL, Williams PG, Meyer BJ (2006). Biomarker validation of a long-chain omega-3 polyunsaturated fatty acid food frequency questionnaire. *Lipids* **41**(9), 845-850.

Sun, Q, J. Ma, et al. (2007). Comparison between plasma and erythrocyte fatty acid content as biomarkers of fatty acid intake in US women. *Am J Clin Nutr* **86**(1), 74-81.

Tanaka T, Shen J, Abecasis GR (2009). Genome-wide association study of plasma polyunsaturated fatty acids in the InCHIANTI Study. *PLoS Genet*. **5,** e1000338.

Teucher B, Skinner J, Skidmore PM. (2007). Dietary patterns and heritability of food choice in a UK female twin cohort. *Twin Res Hum Genet* **10**(5), 734-748.

The National Food Survey available at: www.esds.ac.uk

Theobald HE, Chowienczyk PJ, Whittall R, Humphries SE, Sanders TA. (2004). LDL cholesterol-raising effect of low-dose docosahexaenoic acid in middle-aged men and women. *Am J ClinNutr*. 79(4),558-63.

Thiébaut AC, Rotival M, Gauthier E, *et al*. (2009). Correlation between serum phospholipid fatty acids and dietary intakes assessed a few years earlier. *Nutrition and Cancer* **61**(4), 500-509.

Thompson, FE. and Subar, AF (2001). Chapter 1 - Dietary Assessment Methodology. Nutrition in the Prevention and Treatment of Disease. M. C. Ann, L. R. Cheryl, C. L. R. Elaine R. MonsenA2 - Ann M. Coulston and R. M. Elaine. San Diego, Academic Press**:** 3-30.

Trégouët DA and Garelle V (2007). A new JAVA interface implementation of THESIAS: Testing haplotype effect in association studies. *Bioinformatics*. **23**,1038-1039.

Trégouët D-A, Ducimetère P, et al. (1997). Testing association between candidate-gene markers and phenotype in related individuals, by use of estimating equations. *Am J Hum Genet* **61**:189-19

Tregouet, D. A, S. Escolano, et al. (2004). A new algorithm for haplotype-based association analysis: the Stochastic-EM algorithm. *Ann Hum Genet* **68**(Pt 2), 165-177.

Tregouet, D. A. and V. Garelle (2007). A new JAVA interface implementation of THESIAS: testing haplotype effects in association studies. *Bioinformatics* **23**(8), 1038-1039.

Trégouët, DA, Escolano S, et al. (2004). A new maximum likelihood algorithm for haplotype-based association analysis: the SEM algorithm. *Ann Hum Genet* 68: 165-177.

Truong, H, Dibello, J R, Ruiz-Narvaez, E, Kraft, P, Campos, H & Baylin, A (2009). Does genetic variation in the delta6-desaturase promoter modify the association between alpha-linolenic acid and the prevalence of metabolic syndrome? *Am J Clin Nutr,* **89,** 920-5.

Tunstall-Pedoe H, Vanuzzo D, Hobbs M *et al*. (2000). Estimation of contribution of changes in coronary care to improving survival, event rates, and coronary heart disease mortality across the WHO MONICA Project populations. *Lancet* **355**(9205), 688-700.

Vessby B, Gustafsson IB, Tengblad S, *et al*. (2002). Desaturation and elongation of fatty acids and insulin action. *Ann N Y Acad Sci* **967**, 183-195.

Vessby B, Gustafsson IB, Boberg J, *et al.* (1980). Substituting polyunsaturated for saturated fat as a single change in a Swedish diet: effects on serum lipoprotein metabolism and glucose tolerance in patients with hyperlipoproteinaemia. *Eur J Clin Invest* **10**(3), 193-202.

Vessby B, Uusitupa M, Hermansen K, *et al.* (2001). Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: The KANWU Study. *Diabetologia* **44**(3), 312-319.

Visscher, PM, Hill, WG *et al.* (2008). Heritability in the genomics era--concepts and misconceptions. *Nat Rev Genet* **9**(4), 255-266.

Voight, B F, Peloso, G M, Orho-Melander, M, Frikke-Schmidt, R, Barbalic, M, Jensen, M K, Hindy, G, Holm, H, Ding, E L, Johnson, T, *et al.* (2012). Plasma hdl cholesterol and risk of myocardial infarction: A mendelian randomisation study. *Lancet* **380,** 572-80.

Voruganti VS, Higgins PB, Ebbesson SO, *et al.* (2012). Variants in CPT1A, FADS1, and FADS2 are Associated with Higher Levels of Estimated Plasma and Erythrocyte Delta-5 Desaturases in Alaskan Eskimos. *Front Genet* **3**: 86. doi: 10.3389/fgene.2012.00086.

Warensjö E, Ingelsson E, Lundmark P *et al.* (2007). Polymorphisms in the SCD1 gene: associations with body fat distribution and insulin sensitivity. *Obesity* (Silver Spring) **15**(7), 1732-1740

Warensjö E, Rosell M, Hellenius ML, *et al*. (2009). Associations between estimated fatty acid desaturase activities in serum lipids and adipose tissue in humans: links to obesity and insulin resistance. *Lipids Health Dis* **8**: 37. doi: 10.1186/1476-511X-8-37.

Warrell, D. A, T. M. Cox, et al. (2010). Oxford textbook of medicine. Oxford ; New York, Oxford University Press.

Welch AA, Shakya-Shrestha S, Lentjes MA, Wareham NJ, Khaw (2010). Dietary intake and status of n-3 polyunsaturated fatty acids in a population of fish-eating and non-fish-eating meat-eaters, vegetarians, and vegans and the product-precursor ratio [corrected] of alpha-linolenic acid to long-chain n-3 polyunsaturated fatty acids: results from the EPIC-Norfolk cohort. *Am J Clin Nutr* **92**(5), 1040-1051.

Wennberg M, Vessby B, Johansson (2009). Evaluation of relative intake of fatty acids according to the Northern Sweden FFQ with fatty acid levels in erythrocyte membranes as biomarkers. *Public Health Nutr* **12**(9), 1477-1484.

Whitlock, G, Lewington, S, Sherliker, P, Clarke, R, Emberson, J, Halsey, J, Qizilbash, N, Collins, R & Peto, R (2009). Body-mass index and cause-specific mortality in 900 000 adults: Collaborative analyses of 57 prospective studies. *Lancet*, **373,** 1083-96.

WHO/FAO (2010). Fats and fatty acids in human nutrition. Report of an expert consultation. Rome, Italy. Food and Agricultural Organisation. Available from: http://www.fao.org [Accessed 01/08/2012]

World Health Organization (1999) Definition, Diagnosis and Classificationof Diabetes Mellitus and its Complications. Report of a WHO consultation. Geneva: World Health Organization.

Wrieden W, Peace H. et al (2003). A short review of dietary assessment methods used in national and Scottish research studies. Briefing paper prepared for: working group on monitoring Scottish dietary targets workshop. Available online at (http://www.food.gov.uk/multimedia/pdfs/scotdietassessmethods.pdf)

Xie, L and Innis,SM (2008). Genetic variants of the FADS1 FADS2 gene cluster are associated with altered (n-6) and (n-3) essential fatty acids in plasma and erythrocyte phospholipids in women during pregnancy and in breast milk during lactation. *J Nutr* **138**(11), 2222-2228.

Yli-Jama P, Haugen TS, Rebnord HM, Ringstad J, Pedersen JI (2001). Selective mobilisation of fatty acids from human adipose tissue. *Eur J Intern Med* **12**(2), 107-115.

Zhang B, Wang P, Chen CG, He QQ, Zhuo SY, Chen YM, Su (2010). Validation of an FFQ to estimate the intake of fatty acids using erythrocyte membrane fatty acids and multiple 3d dietary records. *Public Health Nutr* **13**(10), 1546-1552.

Zhou, L and Nilsson, A (2001). Sources of eicosanoid precursor fatty acid pools in tissues. *J Lipid Res* **42**(10), 1521-1542.

Ziegler,A and Konig, I (2006) A statistical approach to genetic epidemiology: concepts and applications. Germany: Wiley-VCH

Zietemann, V, Kröger J, Enzenbach C, Jansen E, Fritsche A, Weikert C, Boeing H, Schulz MB (2010). Genetic variation of the FADS1 FADS2 gene cluster and n-6 PUFA composition in erythrocyte membranes in the European Prospective Investigation into Cancer and Nutrition-Potsdam study. *Br J Nutr* **104**(12), 1748-1759.

Zock PL, Mensink RP, Harryvan J, de Vries JH, Katan MB (1997). Fatty acids in serum cholesteryl esters as quantitative biomarkers of dietary intake in humans. *Am J Epidemiol* **145**(12), 1114-1122.

Zuijdgeest-Van Leeuwen, S D, Dagnelie, P C, Rietveld, T, Van Den Berg, J W & Wilson, J H (1999). Incorporation and washout of orally administered n-3 fatty acid ethyl esters in different plasma lipid fractions. *Br J Nutr*, 82, 481-8.

Appendices

Appendix-1 Macadamia study

A comparison of the differential effects of dietary palmitoleic acid and palmitic acid on markers of cardiovascular health in young healthy men aged 18-45.

Hypothesis

Consumption of palmitoleic acid alters postprandial lipid and glucose metabolism compared with control group

Objectives

To conduct a randomized controlled trial to compare the acute effects on lipid and glucose metabolism of test meals containing palmitoleic acid (macadamia oil; test group) and palmiticc acid (palm oil; control group).

Study design

Ethical approval

Ethical approval was obtained from the Berkshire Research Ethics Committee (Reading, UK) (REC reference 11/SC/0131) on 19/05/2011. Written informed consents were acquired from all participants before starting the study. All clinical procedures were conducted by qualified staff.

Study design

A randomized, single blind, parallel design compared the postprandial response of exchanging palmitoleic acid (12g) with palmtic acid (12g) in the diet. Following a run-in diet for 2-weeks and participants were randomized to one of the two treatment sequences. Each dietary period was to last for one week and was preceded by a test meal. The fat exchanges were achieved by providing subjects specially prepared muffins and a supply of nuts to consume. Subjects received a standardized meal consisting of a muffin (30g test fat) and milk shake (20g test fat) consisting of a total of 50g test fat. Venous blood samples (a total of 80ml) were drawn at regular intervals over 6-hours. The amount of fat exchanged in the two meals of this study was based on changes in LDL-C concentration when exchanging a 12 g of palmitic acid with the

same amount of palmitoleic acid. A 0.24 mmol/l difference in LDL-C concentration is expected between the two groups as calculated by Mensink & Katan equation.

The primary outcome for the test meal was change in triglycerides concentration. Secondary outcome were changes in other blood lipids (HDL-cholesterol, LDL-C and NEFA concentration and fatty acid profiles), C-peptide, CRP, IL-6, glucose and insulin sensitivity (assessed by HOMA).

Test materials and methods

Test meal used

Subjects were asked to avoid high fat food, alcohol as well as participation in any strenuous exercises. Subjects were given a standardized low fat dinner (<10 g fat, Sainsbury's '*Be good to yourself range*') to consume on the day preceding the study day. Postprandial test meal will consist of 1 muffin containing 30g test fat and a milkshake to provide 846 kcal, 20 g fat, 14 g protein and 87g carbohydrates. The milkshake contains 220 ml skimmed milk and 15 g nesquik milkshake mix and were prepared freshly on the day of intervention providing 131 kcal, 7 g protein and 26 carbohydrates. Each muffin contained 15 test fat, 9 g corn flour, 27.5 g wheat flour, 27.5 g white caster sugar, 1.9 g baking powder, 36.9 g skimmed milk, 3.9 g egg white and 3.9 g vanilla essence and to provide 715 kcal, 7 g protein, 62 g carbohydrates and 25 g fat. Additional 10% test fat was added to the muffin recipe to allow for 2.5 g fat loss during baking.

Table.1 Fatty acid composition of the test meals

FA	Test muffin	Control muffin
14:0	1.27	0.59
16:0	8.08	9.53
16:1	16.42	0.28
18:0	3.22	2.91
18:1n-9	54.78	74.84
18:2n-6	6.67	6.90
18:3n-3	0.17	0.25
18:3n-6	0.02	0.02
20:3n-6	0.02	0.03
20:4n-6	0.79	0.64

Test fats used and their properties

Test fats consist of commercially available food grade macadamia oil, high in palmitoleic acid, and food grade palm olein, high in palmitic acid.

Control meal (Postprandial study):

Muffin made with blend of 30g HOS: Palm olein in ratio of 22:8

Milkshake with 100% HOS 20g

Macadamia meal (Postprandial study):

Muffin made with 100% macadamia oil 30g

Milkshake with blend of 20g macadamia: safflower oil blend in a ratio of 17:3

Approximate fat composition:

SFA ~14%, MUFA 79%, PUFA ~7%

Background muffin

80% palm olein, 10% safflower oil, 10% HOS

Composition of the test fats

Table.2 Fatty acid proportions of the control and test fats

FA	Test oil	Control oil
14:0	0.74	0.23
16:0	8.06	9.57
16:1	16.93	0.26
18:0	3.32	3.00
18:1n-9	56.45	77.45
18:2n-6	6.55	6.90
18:3n-3	0.16	0.01
18:3n-6	0.01	0.01
20:3n-6	0.02	0.04
20:4n-6	0.81	0.66

Statistical analysis of data

Data were analysed by repeated measures analysis of variance using GraphPad.

Results

Characteristics of the study participants

A total of 6 subjects were recruited and they only completed the first test meal and did not complete the 3 week dietary intervention. **Table 3** summarizes the characteristics of the study participants.

Table.3 Characteristics of the study male participants stratified by intervention group

	Intervention	Control
	n=3	*n*=3
Age	33.67±8.14	26.67±1.53
Height	185.00±5.20	180.33±5.86
Weight	85.37±18.93	80.53±11.12
Waist	99.50±4.95	94.33±7.51
BMI	24.90±5.20	24.70±2.21
Systolic BP (mmHg)	131.67±5.13	124.33±10.52
Diastolic BP (mmHg)	74.67±2.02	75.17±3.75
Total cholesterol (mmol/L)	5.20±1.56	5.13±0.35
Triacylglycerol (mmol/L)	1.10±0.36	1.20±0.78
LDL-cholesterol (mmol/L)	3.37±1.34	2.90±0.10
HDL-cholesterol (mmol/L)	1.30±0.35	1.67±0.55
TC:HDL-C ratio	4.13±1.16	3.30±0.98
Glucose (mmol/L)	5.50±0.30	5.10±0.20
Insulin (mIU)	7.34±3.53	5.27±1.26

Values are mean ± SD

Postprandial changes in lipids and lipoproteins

Plasma triacylglycerol and fatty acid concentrations

The increase in plasma TAG was lower on the control diet than on the macadamia test meal, but the total fatty acid concentrations did not differ between treatments. The plasma concentration of palmitoleic acid was substantially increased following the macadamia oil test meal. The fall in plasma NEFA was similar following both test meals and there were no differences in postprandial insulin, C-peptide and glucose.

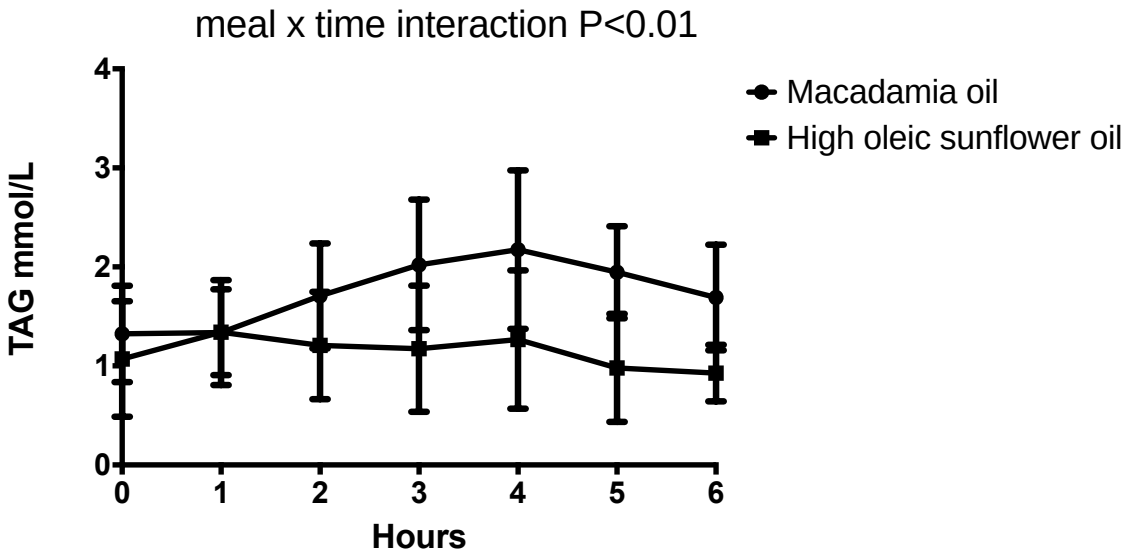

Figure.1 Changes in serum triglycerides.

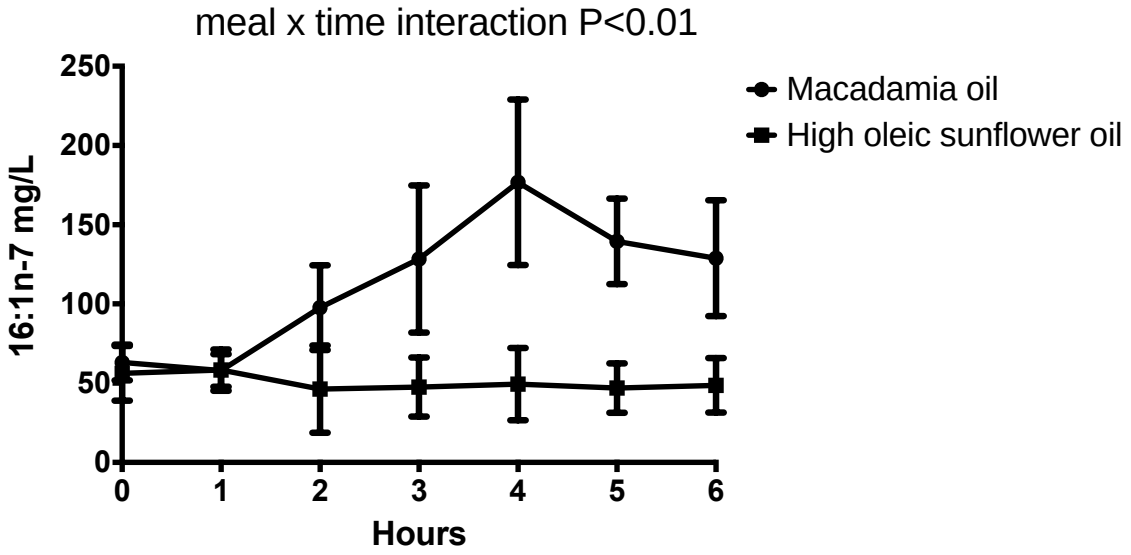

Figure.2 Plasma concentrations of palmitoleic acid**.**

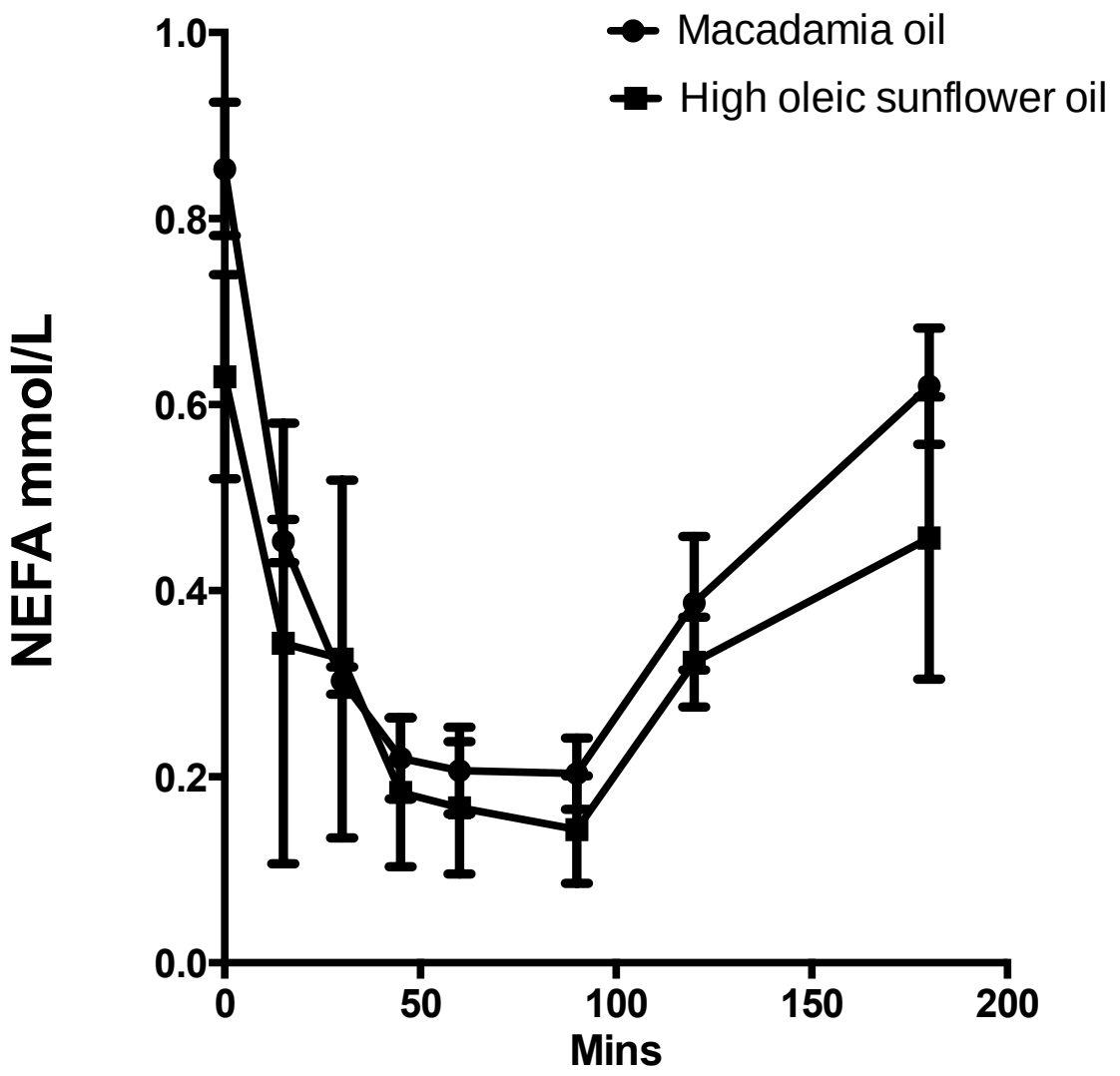

Figure.3 Changes in plasma NEFA concentrations**.**

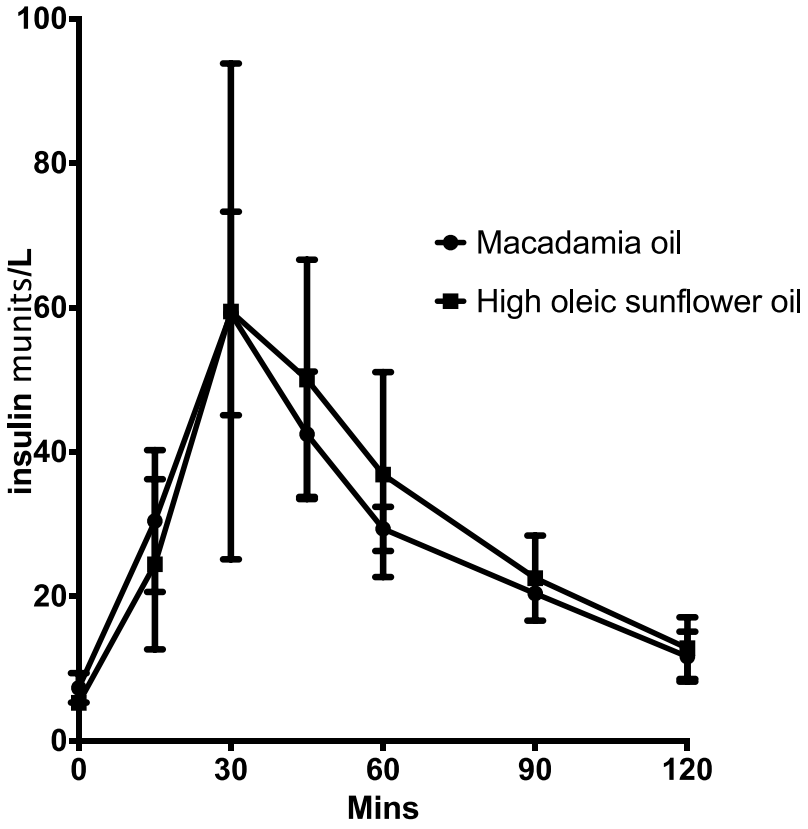

Figure.4 Changes in plasma insulin.

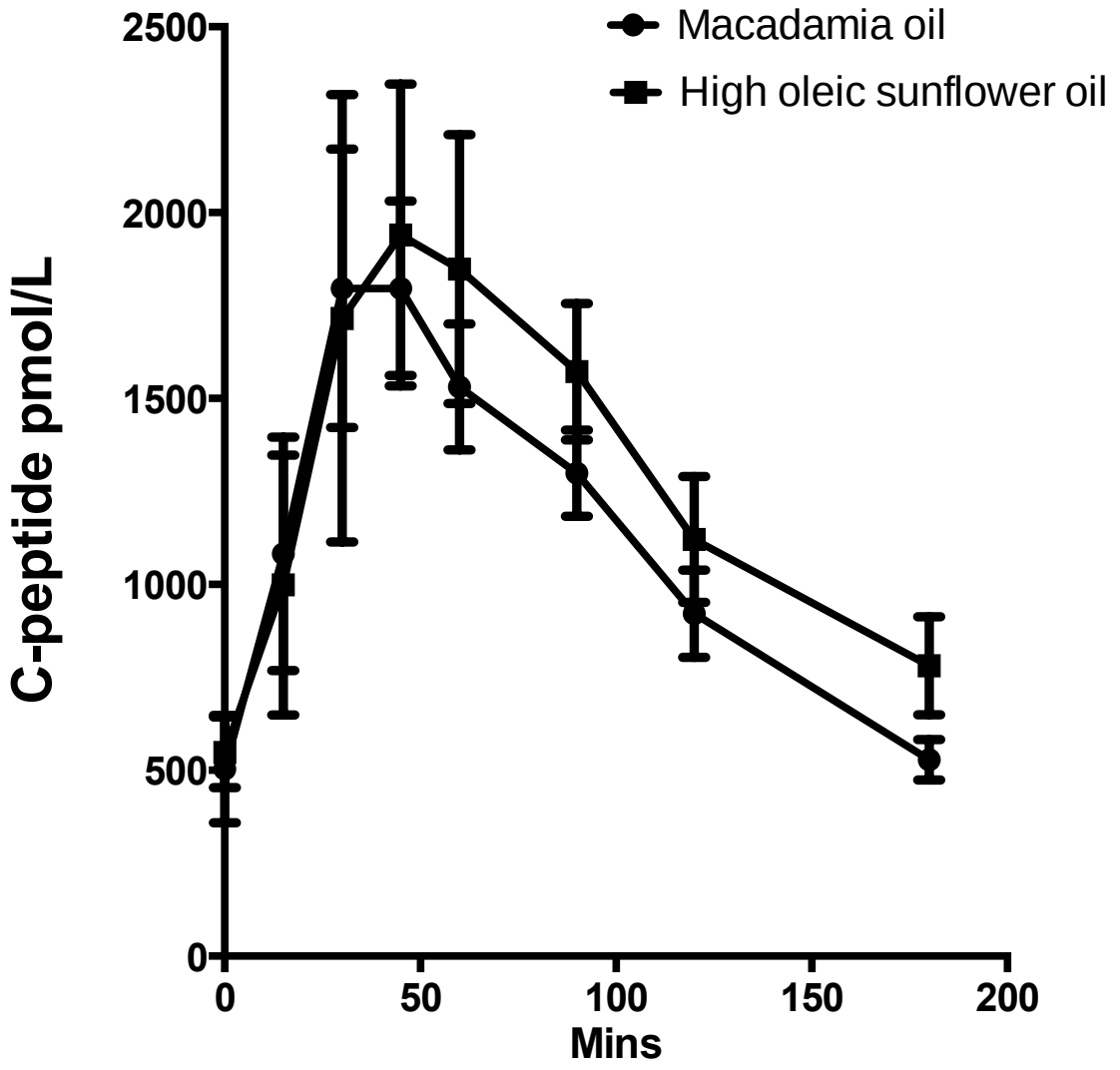

Figure.5 Changes in C-peptide**.**

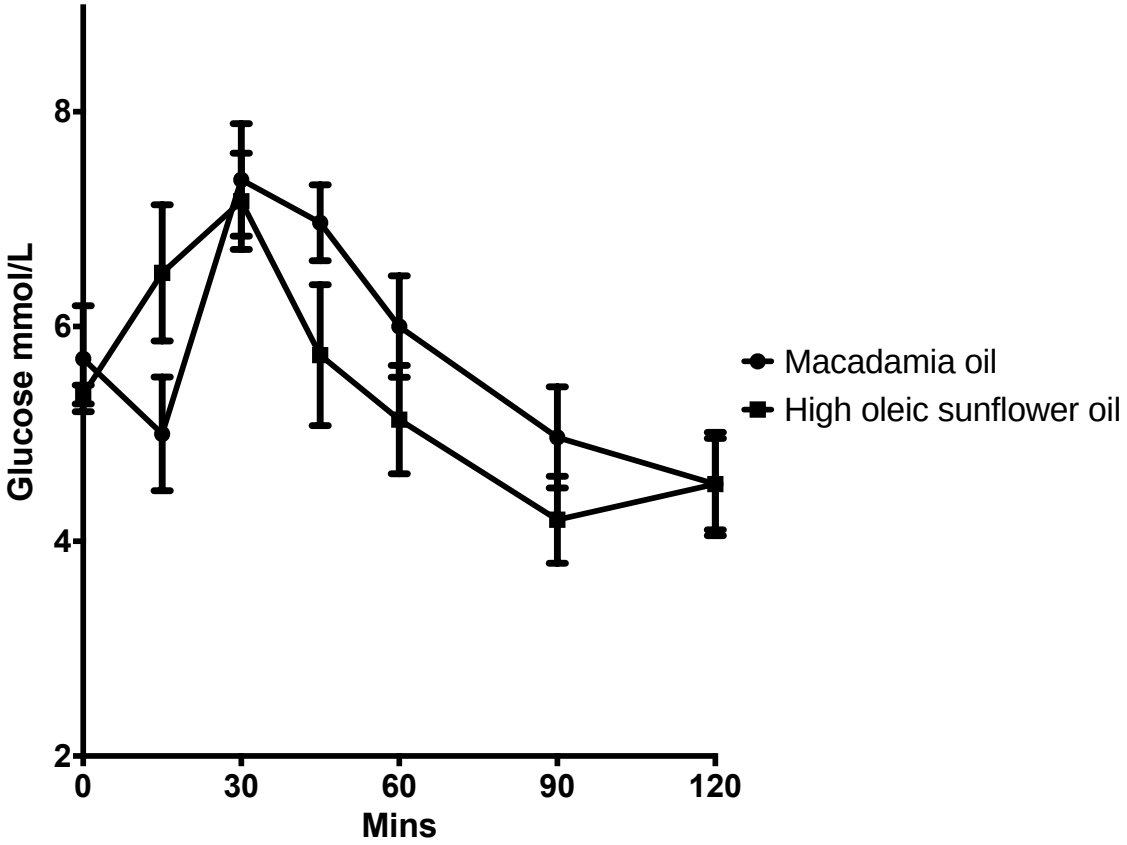

Figure.6 Changes in plasma glucose

INFORMATION SHEET FOR PARTICIPANTS

REC Protocol Number: **11/SC/0131**

YOU WILL BE GIVEN A COPY OF THIS INFORMATION SHEET

The macadamia oil and palm oil trial

We would like to invite you to participate in this original research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Do I have to take part? It is up to you to decide whether to take part or not. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You can withdraw from the study at any time by informing the researcher and you are not obliged to give a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What is the purpose of this study?

The main aim of this study is to investigate the differential effects palmitoleic acid, which is a monounsaturated fatty acid, found in macadamia nut with that of palmitic acid, which is a saturated fatty acid found in many food items. Macadamia oil will be used as a source of palmitoleic acid, which is a monounsaturated fatty acid and palm oil will be used as a source of palmitic acid.

Why have I been chosen?

You have been contacted as you have expressed an interest in our research. In order to participate in this study you need to be able to say 'Yes' to the following:

- I am male
- I am aged between 18 and 45 years
- I do not smoke
- I have never had a heart attack, stroke, high blood pressure (>160/100mm Hg), liver diseases, diabetes, chronic gastrointestinal disorder or cancer
- I do not have a history of excess alcohol intake or substance abuse
- I do not have allergy to either macadamia, hazelnut or palm kernel

- I am prepared to consume the muffins everyday for 10 weeks

What will happen to me if I take part?
Screening: If you would like to participate you would first need to complete a screening questionnaire with us over the telephone (approx. 15 mins), after which potentially eligible volunteers will be invited to attend a clinic screening appointment (approx. 45 mins) in the Metabolic Unit on 4th Floor, Corridor B, Franklin-Wilkins Building, Stamford Street, SE1 9NH. Volunteers will need to attend this visit after an overnight fast.
The study will be explained in detail and you will be able to ask any questions you may have to ensure you will be giving fully informed consent. Following the signing of the consent form, your height, weight and percentage body fat measurements will be recorded, and a fasting blood sample (~3 teaspoons) will be taken to determine whether liver function, haematology, blood glucose and blood lipids are within normal ranges. Seated blood pressure will be measured using an automated sphygmomanometer (blood pressure monitor) that conforms to the recommendation of British Hypertension Society. Refreshments will be provided once all samples and measurements have been made. You will also be asked to complete a food frequency questionnaire.

Participation in the Main Study: Eligible subjects will be asked to complete a 10-weeks period taking 2 muffins per day whilst following dietary advice to avoid high fat food. The muffins will be 'run-in' or 'wash-out' (the same muffins) from weeks 1 to 2 and 6 to 7 of your participation in the study. From weeks 3 to 5 and weeks 8 to 10, the muffins will be 'test' muffins containing either palmitoleic or palmitic acids. We will ask you for a small blood sample at the start of week 3 and week 8. At the end of week 5 and week 10 only on your visit to the Metabolic Research Unit, we would ask you to consume another muffin and a milkshake. You will be cannulated (a tube inserted into the antecubital vein on your arm) for ease of blood withdrawal over a 6 hour post-prandial time. A total of 80mls of blood would be taken over this time – so although it is a large time commitment, the amount of blood is not so great (about 15 teaspoons). Although you will be cannulated for 6 hours, you can freely move. At your four study visits (week 3, 5, 8 and 10) we will also measure your blood pressure, weight and percentage body fat.

To standardise all your visits, you will be asked to avoid strenuous physical activity, foods high in fat, caffeine or alcohol on the day before the visit. We will give you a list of foods to avoid and a list of suggested evening meals to help you with this. Only water will be allowed after 20:00h the evening before the study visits.

Will my participation be kept confidential? Any information collected about you during this research will be kept strictly confidential. Your GP will not be told that you are taking part in the study, unless you request us to do so. Subject confidentiality and anonymity will be observed throughout the study by use of subject codes in place of names, and the storage of subject details in a secure place. Only the investigators have access to this data. Should you wish to find out the results of this study you are welcome to contact us for a copy of the final report once the study is finished.
Possible risks/ benefits from participating the risk associated with the blood collection is small, but there may be a small amount of bruising. All blood collection will be conducted by a train phlebotomist. Furthermore, a trained first aid specialist in the Diabetes and Nutritional Sciences

Division is at hand in any event. You will get your blood levels of cholesterol, triglycerides, glucose and insulin at the end as benefit from participating in the study

What will happen to my study results? We hope to publish the results of the whole study in a scientific journal. You will not be identified in any publication. We will be happy to discuss the overall results with you when the study is completed, and will let you know how you can get a copy of the published results if you wish.

Who is organizing and funding the study? The study is organized and funded by the Diabetes and Nutritional Sciences Division, Kings College London.

If this study has harmed you in any way you can contact King's College London using the details below for further advice and information:

Thank you for your interest.

The contact details for the chief investigator (Maryam Al-Hilal) and her supervisors (Prof Tom Sanders and Dr Anne Mullen) are below. Please do not hesitate to contact any of the research group for further information or suggestions.

Maryam Al-Hilal Tel; email: Maryam.Al_Hilal@kcl.ac.uk; tel: 020 7848 4301

Prof Tom Sanders; email: Tom.Sanders@kcl.ac.uk; tel: 020 7848 4273

Dr Anne Mullen; email: Anne.Mullen@kcl.ac.uk; tel: 020 7848 4081

Diabetes and Nutritional Sciences Division, King's College London, School of Medicine, Franklin Wilkins Building, 150 Stamford Street, London SE1 9NH

NHS
National Research Ethics Service
NRES Committee South Central - Berkshire
Building L27
University of Reading
London Road
Reading
RG1 5AQ

Telephone: 0118 918 0551
Facsimile: 0118 918 0559

19 May 2011

Miss Maryam Al-Hilal
King's College London
150 Stamford Street
Franklin-Wilkins Building Room4.46A
London
SE1 9NH

Dear Miss Al-Hilal

Study title: **A comparison of the differential effects of dietary palmitoleic acid and palmitic acid on markers of cardiovascular health in young healthy men aged 18-45**

REC reference: **11/SC/0131**

Thank you for your letter of 12 May 2011, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

The Committee has not yet been notified of the outcome of any site-specific assessment (SSA) for the non-NHS research site(s) taking part in this study. The favourable opinion does not therefore apply to any non-NHS site at present. We will write to you again as soon as one Research Ethics Committee has notified the outcome of a SSA. In the meantime no study procedures should be initiated at non-NHS sites.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of

Appendix-2 Mx script

Saturated Model

```
! MODEL     :     Phenotypic Univariate Saturated Model, one trait per
twin
! INPUT     :     Raw Continuous Data

#NGroups 3
#define nvar 2                                    ! 2 x 1 trait per
twin

G1: Parameters
Calculation
Begin Matrices;
X Lower nvar nvar Free                    ! Cholesky decomposition for
MZs
Y Lower nvar nvar Free                    ! Cholesky decomposition for
DZs
End Matrices;
Begin Algebra;
M= X*X';                                       ! MZ covariance
N= Y*Y';                                       ! DZ covariance
O=\stnd(M);                               ! MZ correlation
P=\stnd(N);                               ! DZ correlation
End Algebra;
Start 1 X 1 1 1 X 1 nvar nvar               ! starting
values=sqrt(variance)
Start 1 Y 1 1 1 Y 1 nvar nvar       ! starting values=sqrt(variance)
Interval O 2 1                ! 95% CI of MZ twin correlations
Interval P 2 1                ! 95% CI of DZ twin correlations
End

G2: MZF datagroup
Data NInput_vars=5
missing =900
!REC FILE=C:\Documents and Settings\user\Desktop\Mx\Oleic
acid\oleictrial.dat
REC FILE=oleictrial.dat
Labels  famid  age1 c181n9at1  c181n9at2 zyg
SELECT if zyg=1 /
SELECT c181n9at1  c181n9at2  /
Begin Matrices = Group 1;
G Full 1 nvar Free                        ! means
End Matrices;

Start 40 G 1 1 to G 1 nvar                   ! start value for the
means, change here
Means G /                                      ! model for means
Covariances M /                                ! model for MZ
variance/covariances
Options RSiduals
End

G3: DZF datagroup
Data NInput_vars=5
missing =900
```

```
!REC FILE=C:\Documents and Settings\user\Desktop\Mx\Oleic
acid\oleictrial.dat
REC FILE=oleictrial.dat
Labels  famid  age1 c181n9at1   c181n9at2 zyg
SELECT if zyg=2 /
SELECT  c181n9at1  c181n9at2 /
Begin Matrices = Group 1;
G Full 1 nvar Free                        ! means
End Matrices;
Start 40 G 1 1 G 1 nvar                       ! start value for the
means, change here
Means G /                                       ! model for means
Covariances N /                                 ! model for DZ
variance/covariances
Options RSiduals
End
```

ACE Model

```
! Univariate Script for Raw data anlayses
#define nvar 1
G1: DEFINE MATRICES
CALCULCATION NGROUPS=3
BEGIN MATRICES;
X LOWER nvar nvar  FREE           !Path coefficients genetic
Y LOWER nvar nvar  FREE           !Path coefficients shared env
Z LOWER nvar nvar  FREE           !Path coefficients unique env
END MATRICES;
BEGIN ALGEBRA;
A=X*X';
C=Y*Y';
E=Z*Z';
P=A+C+E;
S=A%P|C%P|E%P;         ! h2|c2|e2
END ALGEBRA;
MA X 1                       ! start value= sqrt (variance/3)
MA Y 1                       ! start value= sqrt (variance/3)
MA Z 1                       ! start value= sqrt (variance/3)
OPTION RS
!Interval S 1 1 S 1 2 S 1 3       ! 95% CI for h2, c2, e2
END

G2: MZF Group
Data NInput_vars=5
missing =900
!REC FILE=C:\Documents and Settings\user\Desktop\Mx\Oleic
acid\oleictrial.dat
REC FILE=oleictrial.dat
Labels  famid  age1 c181n9at1  c181n9at2   zyg
SELECT if zyg=1 /
SELECT c181n9at1  c181n9at2  /
MATRICES = GROUP 1
M full 1 2 FREE
COVARIANCE
 ( A + C + E | A + C _
```

```
   A + C     | A + C + E ) /

MEANS M /

MA M 40 40             ! start value for the means, change here
OPTION RS
END

G3: DZ Group
Data NInput_vars=5
missing =900
!REC FILE=C:\Documents and Settings\user\Desktop\Mx\Oleic
acid\oleictrial.dat
REC FILE=oleictrial.dat
Labels  famid  age1 c181n9at1  c181n9at2   zyg
SELECT if zyg=2 /
SELECT c181n9at1  c181n9at2  /

MATRICES = GROUP 1
M FULL 1 2 FREE
H FULL 1 1

COVARIANCE
 ( A + C + E | H@A + C _
   H@A + C   | A + C + E ) /

MEAN M /

MATRIX H .5

MA M 40 40             ! start value for the means, change here
opt rs
Options Multiple
End

Save ace.mxs           !this saves the full model so you can test
reduced models

Get ace.mxs
drop X 1 1 1                !this drops the a term - tests CE
model
end group;

get ace.mxs
drop Y 1 1 1                !this drops the c term - tests AE
model
end group;

get ace.mxs
drop X 1 1 1 Y 1 1 1        !this drops the a and c from ACE -
tests E model
end group;
```

Appendix-3 CRESSIDA study

Dietary intervention

Reduction in SFA:

A lower SFA intake at 10% was achieved in the high MUFA diet at the same time total fat intake sustained at 35%.

Removal of as much dairy fat as possible.

Providing the participants with oils and spreads low in SFA.

Advice to select lean meat (restricting lean meat intake to ≤3 servings/week).

Increasing proportion of *n*-6:

Achieved by exchanging some of the oleic acid with linoleic acid.

Substitution of fat with varying composition mainly oils, spreads, dairy foods and reformulated products.

Increasing long-chain *n*-3 PUFA:

Encouraging participants to increase oily fish intake.

Reimbursing the subjects for cost of oily fish purchased.

Supplying the participants with tinned oily fish and other preserved fish products.

Increasing wholegrain and cereals intake:

Provide the subject with a choice of breakfast cereals, which are either high or low in wholegrain cereals.

Advice participant to select wholegrain cereals where there has been minimum disruption to the grain as opposed to highly processed wholegrain items.

Advice subjects to consume wholegrain bread.

Provide participants with packets of microwavable brown rice or long-grain white rice.

Recommend that the potatoes are prepared in a way so that they are not high in fat and the potassium content is retained (baked, microwaved, or oven chips).

Reduction in salt intake:

Avoidance of food high in salt.

The use of reduced salt options.

Reduction in the discretionary salt use.

Provide the participant with advice to avoid foods high in salt (bacon, olives, some ready prepared products such as soups, ready meals that contain >2 g salt/serving).

Provide the subject with reduced salt alternatives for tomato sauce and baked beans or conventional versions of the same product.

Increasing fruit and vegetables:

Participants were advised to consume 5 portions of fruits and vegetables/day.

Consumption of wide variety of fresh fruit and vegetables was encouraged.

Subjects were provided with sample menus and preparation instructions in order to retain the maximum nutritional value (i.e. cooking in minimum volume of water and avoiding exposure of cut vegetable to air, light or heat for long periods of time).

Participants were discouraged from the consumption of fruit in syrup or vegetable to which salt (i.e. pickled foods and olives) or fat (i.e. coleslaw, sun-dried tomatoes in oil) was added.

Fruit juice counted for one portion only according to the Department of Health advice.

Study participants

Exclusion criteria

A reported history of angina pectoris, myocardial infarction, stroke, peripheral vascular disease or congenital heart disease.

Asymptomatic atrial fibrillation.

Type 1 or Type 2 diabetes mellitus (fasting blood glucose > 7.0mmol/L).

Seated blood pressure > 160/105mmHg.

Current use of medication for lowering blood cholesterol (statins) or blood pressure.

Body Mass Index <18.5 and >35kg/m2.

An overall risk of cardiovascular disease over the next ten years of >20% assessed according to current NICE guidelines plus untreated high blood pressure or raised cholesterol

Clinical history of cancer (excluding basal cell carcinoma) in the past five years.

Chronic renal, liver or inflammatory bowel disease.

Current cigarette smoking (confirmed by urinary cotinine analysis – value > 500ng/L indicates current smoking).

History of substance abuse or alcoholism (previous weekly alcohol intake >60 units/men or 50 units/women).

Current self-reported weekly alcohol intake exceeding 21 units for women and 28 units for men.

Currently pregnant, planning pregnancy or having had a baby in the last 12 months.

Unwilling to follow the protocol and/or give informed consent.

Unwilling to refrain from use of dietary supplements.

Unwilling to restrict or increase consumption of oily fish.

Weight change of >3kg in preceding 2 months.

Exclusion drugs

Haemostatic drugs such as Anti-coagulants - e.g. Warfarin (but do not exclude those taking aspirin).

Medication for lowering blood cholesterol – e.g. statins.

Medication for lowering blood pressure – e.g. ACE inhibitor, Ca channel blocker, thiazide diuretic.